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APRIL 1956, VOLUME 62, No. 4

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CAMBRIDGE UNIVERSITY PRESS
LONDON: BENTLEY HOUSE, N.W.1
NEW YORK: 32 EAST 57TH STREET, 22

*Price 20s. net (U.S.A. $3.25)*

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The fluorimetric determination of $\beta$-glucosidase: its occurrence in the tissues of animals including insects. By D. Robinson

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# Studies on the Origin of Milk Fat

## 1. THE LOCATION OF TRITIUM IN STEARIC ACID PRODUCED BY THE CATALYTIC ADDITION OF TRITIUM TO ELAIDIC ACID

By R. F. GLASCOCK and L. R. REINIUS*
*The National Institute for Research in Dairying, University of Reading*

(*Received* 23 *July* 1955)

Schoenheimer & Rittenberg (1935) assumed that a specifically labelled stearic acid would result from the catalytic addition of deuterium to linoleic acid over a catalyst made from platinum oxide. It was therefore hoped that the catalytic addition of tritium to elaidic acid under somewhat milder conditions (room temperature, over a palladium-black catalyst at less than atmospheric pressure) would result in stearic acid labelled in the 9:10-position. This material was required primarily for work on the contribution of long-chain fatty acids to milk constituents. In a preliminary experiment on a goat (Duncombe & Glascock, 1954), carried out with tritiostearic acid prepared from oleic acid, it was found that steam-volatile fatty acids, glyceride glycerol, lactose and cholesterol isolated from the milk had low but significant specific activities. If the stearic acid administered was specifically labelled these results therefore implied extensive degradation.

It was also found that the specific activities of the saturated and unsaturated fractions of the glyceride fatty acids were about the same. This might have meant a marked isotope effect, hydrogen being removed in preference to tritium during the biological conversion of stearic acid into oleic acid. Before proceeding with further work it thus became necessary to ascertain whether or not the stearic acid used was specifically labelled.

The degradation of stearic acid in such a manner as to determine whether or not the tritium in it is attached to the $C_{(9)}$ and $C_{(10)}$ atoms is a matter of considerable technical difficulty. Methods are available for the degradation of $^{14}C$-labelled fatty acids, but they are not readily applicable to this problem. Thus Hunter & Popják (1951) describe a method for the splitting from long-chain fatty acids of two carbon atoms at a time as acetic acid. The whole procedure involves five steps, and even if applicable to the determination of the location of hydrogen isotopes would obviously present an enormous task if applied to stearic acid. Presumably some such method would have to be used, however, if an exact map of the isotopic distribution in stearic acid were required. For our work, however, it was necessary only to know whether all the tritium resided on the $C_{(9)}$ and $C_{(10)}$ atoms and, if not, how much resided elsewhere on the chain.

In the preliminary biological work already referred to it had been found that after feeding the labelled stearic acid to a lactating goat, the unsaturated fatty acid fraction of the milk had a high specific activity suggesting the biological conversion of stearic acid into oleic acid. If the tritium in this oleic acid were attached to the $C_{(9)}$ and $C_{(10)}$ atoms thus:

$$CH_3.[CH_2]_7.C^3H{:}C^3H.[CH_2]_7.CO_2H$$

then the following series of reactions should yield non-radioactive pelargonic and azelaic acids by labilization at stage (3) of the hydrogen originally on the $C_{(9)}$ and $C_{(10)}$ atoms. This paper deals with the results obtained on submitting the milk oleic acid to this degradation scheme.

$$CH_3.[CH_2]_7.C^3H{:}C^3H.[CH_2]_7.CO_2H \ (1) \xrightarrow[H.CO_2H]{H_2O_2} CH_3.[CH_2]_7.C^3H(OH).C^3H(OH).[CH_2]_7.CO_2H$$

$$(2) \xrightarrow[+\,Pb_3O_4]{CH_3.CO_2H} \begin{cases} CH_3.[CH_2]_7.C^3HO \xrightarrow{O_2} CH_3.[CH_2]_7.CO_2H \\ O^3HC.[CH_2]_7.CO_2H \xrightarrow[(3)]{KMnO_4} HO_2C.[CH_2]_7.CO_2H \end{cases}$$

## EXPERIMENTAL

*Preparation of tritium-labelled stearic acid.* Elaidic acid, the *trans*-isomer of oleic acid, was used as the starting material in this preparation because it is available in pure crystalline form. The technique for adding tritium to the elaidic acid molecule was that already used for the preparation of tritiohexoestrol (Glascock & Reinius, 1954; Glascock & Pope, unpublished work). It was found that 96% of the

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theoretical volume of tritium-hydrogen (nominal specific activity, 15·5 mc/ml.) was taken up by 14·4 mg. of elaidic acid in 68 hr. The product was extracted from the reaction vessel with ether, converted into the sodium salt and re-precipitated to displace tritium that might have entered the carboxyl group. It was pure white, with m.p. 71°; (Heilbron & Bunbury (1943) give 71·5–72°). The yield was 13·4 mg. (93%).

*Assay.* The whole of the product was dissolved in arachis oil, giving a total weight of solution of 1·027 g., and an 8·5 mg. sample was submitted to wet combustion and assayed for tritium as described by Glascock (1954). The oil solution was found to have a specific activity of $5{\cdot}047 \times 10^6$ counts/min./mg., corresponding to $3{\cdot}845 \times 10^8$ counts/min./mg. for the stearic acid dissolved in it. From this result it is calculated that the tritium-hydrogen used must have had a specific activity of 6·6 mc/ml. instead of the nominal 15·5 mc/ml.

*Administration to goat.* The goat was a British Saanen, 3 years old, weight 61 kg., in her second lactation and yielding 2·5 kg. of milk/day. She was fed on stock cereal diet with hay *ad lib.* and kept in a pen during the period of the experiment. A portion of the oil solution of [$^3$H]stearic acid described above, weighing 0·78 g. and containing $3{\cdot}94 \times 10^9$ counts/min., was emulsified in 50 ml. of 5% (w/v) glucose solution together with 0·5 g. of glyceryl monostearate. This emulsion was administered quantitatively by mouth followed by 50 ml. of a similar but non-radioactive emulsion.

*Milking.* The goat was milked at intervals and milk samples were fractionated for the determination of the distribution of radioactivity in various constituents. This work is the subject of part of the next paper (Glascock, Duncombe & Reinius, 1956). Two milk samples, *A* and *B* (vol. 1370 and 820 ml. respectively), collected at 23 and 31 hr. after dosing, were used for the present work.

*Extraction of total fat.* The method used was that of McClymont (1951). The milk was centrifuged at 200 rev./min. for 1 hr., cooled in ice, and the cream removed into a beaker. To the cream was added an equal volume of ethanol containing 10% (v/v) of concentrated $NH_3$ solution (sp.gr., 0·880); the mixture was heated to boiling and then cooled. It was extracted with $3 \times 25$ ml. of a mixture of equal vol. of light petroleum (b.p. 40–60°) and ether. The combined extracts were dried overnight with anhydrous $Na_2SO_4$ and filtered and the solvent was distilled off.

The residue of mixed lipids was transferred to centrifuge tubes with the help of a small amount of light petroleum and the phospholipids were precipitated, according to the method of Bloor (1929), with acetone and $MgCl_2$. The precipitate was centrifuged off and the supernatant solution removed and evaporated to dryness.

Ethanolic KOH (1 vol. of 40% w/v KOH to 10 vol. of absolute ethanol) was added to the fat in the proportions of 50 ml. to 1 g. of fat, and the mixture was refluxed for 3 hr. The solution was then extracted with $5 \times 10$ ml. of light petroleum to remove unsaponifiable material, and the extracted solution was heated on the steam bath to remove organic solvents, water being added towards the end to keep the soaps in solution. It was allowed to cool, and the fatty acids were liberated by the addition of a 10% excess of sulphuric acid (40%, v/v). Volatile fatty acids were then removed by steam distillation until more than the initial volume of solution had been distilled over.

*Separation of unsaturated fatty acids.* The method of Twitchell (1921) was used. The liquid in the flask was allowed to cool and the aqueous lower layer removed. The mixture of fatty acids was dissolved in 5 times its weight of 95% (v/v) ethanol containing 1·5% (v/v) glacial acetic acid. The mixture was heated to boiling, and an equal volume of boiling lead acetate (14% w/v in 95% ethanol containing 1·5% glacial acetic acid) was added. The mixture was allowed to cool to room temperature and then filtered. The filtrate, which contained the unsaturated fatty acids, was tested for an excess of lead acetate by the addition of an ethanolic solution of $H_2SO_4$.

The ethanol was then removed by distillation under reduced pressure and the residue dissolved in ether. The ethereal solution was washed first with water and then with dilute HCl, and again with water until free from mineral acid. The solution was dried over $Na_2SO_4$ and the solvent removed. The residue of unsaturated fatty acids was dried to constant weight by heating gently under vacuum. Yield, 4·7 g. from sample *A*, and 2·7 g. from sample *B*.

*Dilution with carrier.* Both samples of mixed unsaturated fatty acids had specific activities of the order of $10^4$ counts/min./mg. of combustion water. Since specific activities of the order of 100 counts/min./mg. of combustion water can be conveniently measured by the methods of assay used (the gas counters used will hold butane equivalent to 10 mg. of water) it was decided to use carrier oleic acid. The oleic acid used was a laboratory reagent labelled 'oleic acid, redistilled'. Its iodine value was measured and found to be 88·4, which is close both to the theoretical figure (91·5) and to the value on the bottle. The results obtained (see below), however, gave reason to believe that it was not pure, and hence the exact amount of carrier oleic acid used is not known.

Sample *A* was diluted by a factor of 5·14, and sample *B* by a factor of 9·92.

### *Preparation of dihydroxystearic acid*

The method used was that of Swern, Billen, Findley & Scanlan (1945), which is based upon the oxidation of oleic acid with performic acid.

*Sample* A. To 6·578 g. of diluted unsaturated fatty acids were added 50 ml. of formic acid (B.D.H., 98–100%) and 3 ml. of $H_2O_2$ (B.D.H., 98–100 vol.). The mixture, cooled in a cold-water bath, was stirred for 30 min. after the addition of the $H_2O_2$, and then transferred to a bath at 40°. The mixture was continually stirred over a period of 6 hr., $4 \times 3$ ml. portions of $H_2O_2$ being added at 1 hr. intervals. The reaction mixture was poured into 4 vol. of water, and the formoxy derivatives of dihydroxystearic acid were extracted with ether. The ethereal solution was washed with water and the ether was removed by distillation.

To the residue was added 3N-KOH until the mixture was alkaline. It was then boiled under reflux for 1 hr. to hydrolyse the formoxy derivatives, poured into an excess of 3N-HCl, cooled, and the crude dihydroxystearic acid, a white solid, separated from the aqueous layer, which was discarded.

The dihydroxystearic acid was recrystallized once from hot ethanol (5 ml. of ethanol to 1 g. of dihydroxystearic acid), yielding white crystals (2·331 g.), m.p. 88°. Recrystallization of a portion gave a product with the same m.p. [Swern *et al.* (1945) found m.p. 92–94°].

*Sample* B. The procedure was exactly as described for sample *A* except that 4·047 g. of diluted unsaturated fatty acids dissolved in 36 ml. of formic acid was used and oxidized with a total of 10 ml. of $H_2O_2$ added in 2 ml. portions. The product, twice recrystallized, melted at 89–90°, and the yield was 1·468 g.

### *Degradation of dihydroxystearic acid*

*Dilution.* The specific activities of the dihydroxystearic acid samples were expected to be of the order of 2000 counts/min./mg. Only relatively small quantities were available (2·3 g. of sample *A* and 1·47 g. of sample *B*) and the proposed degradation was a two-stage one, giving rise to four different products: pelargonic aldehyde, pelargonic acid, azelaic semialdehyde and azelaic acid. It was therefore decided to dilute the samples about fivefold with carrier. For this purpose dihydroxystearic acid (obtained from L. Light and Co.) was recrystallized once from 95% (v/v) ethanol (m.p. 90°), and used to dilute the radioactive dihydroxystearic acid. The dilutions were: sample *A*: 2·276 g. was diluted to 9·688 g. (dilution factor = 4·26); sample *B*: 1·430 g. was diluted to 5·731 g. (dilution factor = 4·01).

The methods of degradation used were those of Scanlan & Swern (1940).

*Fission of dihydroxystearic acid molecule at the* 9:10-*position.* Using sample *A*, 9·688 g. of diluted dihydroxystearic acid was melted and 76·0 g. of glacial acetic acid added with stirring, the mixture being maintained at 55–60°. $Pb_3O_4$ (23·20 g.) was then added in about 15 small portions of equal size, each portion being added only when the colour from the previous portion had disappeared. The whole reaction time was 3 hr. When all the $Pb_3O_4$ had been added, the solution was tested for excess of lead tetra-acetate with leucomalachite green. The mixture now contained pelargonic aldehyde and azelaic semialdehyde, only the pelargonic aldehyde being volatile in steam. The mixture was therefore distilled in steam until no more oil came over. This was separated from the water and part was converted into the dimedone derivative and part set aside for oxidation to pelargonic acid.

*Pelargonic aldehyde dimedone derivative.* Pelargonic aldehyde (0·250 mg.) and dimedone (600 mg.) were dissolved in 30 ml. of 50% (v/v) ethanol, and the solution was warmed and left standing overnight. White needles which separated on recrystallization from ethanol had m.p. 86° [Staff of the Research Laboratory of Hopkin and Williams Ltd. (1944) give 86°].

*Pelargonic acid.* The remaining pelargonic aldehyde was taken up in 50 ml. of ether and the solution was washed with $NaHCO_3$ until neutral. It was diluted to 100 ml. in a 100 ml. measuring cylinder and oxygen was bubbled through it for 2 hr. The solution was dried over $Na_2SO_4$ and filtered, and the ether removed by distillation. The solution of crude pelargonic acid was made alkaline with N-KOH to displace the hydrogen attached to the carboxyl group and reprecipitated with 2N-HCl. The pelargonic acid was taken up in ether, the ether solution washed with water and the ether then removed by distillation.

*Pelargonic acid* p-*toluidide.* To 509·4 mg. of crude pelargonic acid was added 0·34 ml. of thionyl chloride and the mixture was heated to 110–120° for 30 min. Then 0·345 g. of *p*-toluidine was added, and the mixture was heated at 140° for 30 min. The product was cooled and recrystallized twice from benzene–ethanol mixture (50%, v/v). It had m.p. 83–84° [Heilbron & Bunbury (1943) give 84°].

*Azelaic semialdehyde.* The liquid remaining in the flask after the removal of the pelargonic aldehyde by steam distillation was cooled and extracted with ether. The ether was washed thoroughly with water, filtered and dried over $Na_2SO_4$. The solution was filtered and the ether removed by distillation under reduced pressure. The crude azelaic semialdehyde remained as a yellow oil (yield 2·5 g.). It was not purified but converted into the following derivative.

*Azelaic aldehyde semicarbazone.* A solution of 4·05 g. of semicarbazide hydrochloride and 5 g. of hydrated sodium acetate in 10 ml. of water was prepared, and 1·5 g. of crude azelaic semialdehyde was added to 3 ml. of this solution. The derivative began to crystallize within a few minutes. After standing overnight the crystals were filtered off and recrystallized from aqueous ethanol. They had m.p. 161–162° [Heilbron & Bunbury (1943) give 163°].

*Azelaic acid.* The remainder of the azelaic semialdehyde, amounting to about 1 g., was refluxed with 25 ml. of 0·05N-NaOH and $KMnO_4$ solution (620 mg. of $KMnO_4$ in 5 ml. of water) was added a little at a time. Refluxing was continued until the colour of the permanganate was discharged. The solution was filtered, the combined filtrate and washings were concentrated to about 20 ml., and 2N-HCl was added until the solution was acid. The white precipitate of azelaic acid which formed was filtered off, washed with water and recrystallized from water. It had m.p. 104° [Heilbron & Bunbury (1943) give 106·5°].

The dihydroxystearic acid prepared from sample *B* was degraded in exactly the same way as that from sample *A* except that a smaller quantity (5·73 g.) was used, and the amounts of reagents required were proportionately less. The same derivatives were prepared, and found to have the same melting points.

### *Determination of tritium*

Tritium was determined by combustion of 5–10 mg. samples of the derivatives and conversion of the combustion water into butane, followed by gas counting as described by Glascock (1951, 1952, 1954).

## RESULTS

The original unsaturated fatty acid fractions from milk samples *A* and *B* were found to have specific activities of 10 800 and 10 200 counts/min./mg. of combustion water, and were diluted with carrier oleic acid by factors of 5·14 and 9·92 respectively. If both the milk unsaturated fatty acids and the carrier were pure oleic acid, the mixtures should thus have had specific activities of 2100 and 1030 counts/min./mg. of combustion water. The specific activities of the dihydroxystearic acid samples prepared from these oleic acid dilutions (after correction for the further dilution carried out at the dihydroxystearic acid stage) were, however, 3250 and 1470 counts/min./mg. of water. Corrected to the hydrogen content of oleic acid itself, these figures correspond to 3440 and 1555 counts/min./mg. of oleic acid combustion water. They are about

50 % higher than would have been expected if both carrier and milk unsaturated fatty acids were pure oleic acid, but they are in about the same ratio to each other as would then have been expected.

These higher values could not arise only from the milk unsaturated fatty acids being composed partly of acids other than oleic: the carrier oleic acid must also have been impure. The specific activity data obtained and the dilutions actually used would fit if the carrier was about 60 % oleic acid and the milk unsaturated acids about 65 % oleic acid. It is obvious, however, that the proportion of oleic acid in the milk acids cannot be calculated exactly if the purity of the carrier is not known, and this value is neither known nor easily ascertainable.

contained in the pelargonic aldehyde as in the azelaic semialdehyde. Since no loss of tritium had occurred during the fission of the molecule it seems unlikely that this is an analytical artifact; it indicates that about 68 % more tritium was attached to the carbon atoms on the methyl side than on the carboxyl side of the double bond of the original oleic acid.

Table 2 shows the specific activities of the two aldehydes and of the corresponding acids produced from them on oxidation. The loss of activity from the azelaic semialdehyde represents the tritium originally attached to the $C_{(9)}$ atom of the original oleic acid, and the loss of activity from the pelargonic aldehyde represents the tritium originally attached to the $C_{(10)}$ atom. The agreement between

Table 1. *Specific activity of dihydroxystearic acid and its decomposition products*

| Substance | Specific activity (counts/min./μmole) Sample *A* | Sample *B* |
|---|---|---|
| Dihydroxystearic acid | 248·0 | 119·0 |
| Pelargonic aldehyde | 152·0 | 81·0 |
| Azelaic semialdehyde | 93·0 } 245·0 | 47·5 } 128·5 |
| Ratio: Specific activity of pelargonic aldehyde / Specific activity of azelaic semialdehyde | 1·64 | 1·72 |

Table 2. *Diminution in specific activity on oxidation of pelargonic aldehyde and azelaic semialdehyde to corresponding acids*

| Substance | Specific activity (counts/min./μmole) Sample *A* | Sample *B* |
|---|---|---|
| Pelargonic aldehyde | 152·0 | 81·0 |
| Pelargonic acid | 131·0 | 61·0 |
| Difference (%) | 14 | 25 |
| Mean difference (%) | 20 | |
| Azelaic semialdehyde | 93·0 | 47·5 |
| Azelaic acid | 83·0 | 45·0 |
| Difference (%) | 11 | 5 |
| Mean difference (%) | 8 | |

The specific activity of each sample of dihydroxystearic acid, and the specific activities of the two aldehydes to which each gives rise on fission, are given in Table 1, the results being expressed in counts/min./μmole for better comparison. It will be seen that, within the limits of experimental error, the activity originally contained in the dihydroxystearic acid was recovered in the decomposition products. It is evident, therefore, that the tritium was stably bound to the dihydroxystearic acid, none being lost by exchange even during treatment with a vigorous oxidizing agent rich in hydrogen ions. It is also to be noted that on the average there was 1·68 times as much activity

the duplicates is poor, as is to be expected when figures are obtained by difference. However, taking the values found, the loss of activity corresponds to an average of 3 % from the $C_{(9)}$ atom of the original oleic acid and 12 % from the $C_{(10)}$ atom. Although it is clear that these figures are only approximate, the conclusion can justifiably be drawn that more tritium resides on the $C_{(10)}$ atom than on the $C_{(9)}$ atom.

## DISCUSSION

The fact that the dihydroxystearic acid samples were radioactive formally proves the biological conversion of stearic acid into oleic acid in the goat. This is in agreement with what has been found for body fat in the rat using deuterium (Stetten & Schoenheimer, 1940).

It is evident from the specific activities of the degradation products that the major part of the tritium, probably as much as 85 %, contained in the dihydroxystearic acid resided on carbon atoms other than the $C_{(10)}$ and $C_{(9)}$. The two main questions that arise are therefore: (1) to what extent does this distribution of tritium reflect that in the oleic acid extracted from the milk fat; (2) to what extent does it reflect the isotope distribution in the original stearic acid administered to the goat?

The distribution of tritium in the dihydroxystearic acid is almost certainly the same as that in the oleic acid from which it was prepared. If it were not, then the labilization that must accompany redistribution would also have resulted in loss of tritium into the reagent, performic acid, which is a substance rich in exchangeable hydrogen. Although, for reasons already given, an exact radioactivity balance was not possible because of the impurity of the carrier oleic acid, the specific activity of the dihydroxystearic acid was higher, not lower, than expected. Furthermore, in the next stage of degradation (dihydroxystearic acid to pelargonic and azelaic aldehydes) a radioactivity balance was achieved after the use of an equally vigorous and somewhat similar oxidizing agent (lead tetraacetate in glacial acetic acid).

Granted, therefore, that the tritium in the oleic acid was stably bound, it is virtually certain that a redistribution of tritium in the molecule had not occurred biologically because the operation of dehydrogenating enzyme systems would result in loss of tritium into coenzyme pools or body water, and not in reattachment to the original molecule in a different position. It therefore follows that the tritium in the original stearic acid was not confined to the $C_{(9)}$ and $C_{(10)}$ atoms and distribution down the chain must have occurred during its preparation.

That this is not only possible but probable is indicated by the findings of other workers investigating the hydrogenation of fats and fatty acids generally. This work, ably surveyed by Markley (1947), indicates that there is an appearance of double bonds in positions other than the original position during the catalytic hydrogenation of ethenoid acids or their esters. Hilditch & Vidyarthi (1929) found that the partial hydrogenation of methyl oleate gives rise to methyl esters of oleic acids with the double bond in both the 8:9- and the 10:11-positions. Moore (1919) hydrogenated ethyl oleate with palladium and nickel catalysts and found esters of monoethenoid acids in the product with the double bond in the 10:11- and in the 11:12-positions. It thus seems highly probable that in the stearic acid used in the work described in this paper the tritium resided also on these positions.

One would, however, still expect more tritium to be located in the 9:10-positions than was actually found. As already noted, the total amount of tritium in these positions was approximately 15% of the total in the molecule, corresponding to 28% in the original stearic acid if no isotope effect occurred during biological conversion into oleic acid. Neglecting the fact that the 9:10-position is most rapidly hydrogenated (Pigulevskii & Artamonov, 1942; quoted by Markley, 1947), and supposing that the isotope were distributed evenly over the 8, 9, 10, 11 and 12 positions, one would expect the 9:10-positions together to account for 40% of the tritium in the molecule. The difference between this figure and ours (28%) is too great to be due to experimental error and must be due to some other cause. One possibility is that the stearic acid dehydrogenating system may have labilized all the tritium atoms on the $C_{(9)}$ and $C_{(10)}$ atoms, resulting in the loss of more than half as would be expected: i.e. half is lost by dehydrogenation followed by a further loss through exchange with unlabelled hydrogen in the body water. Fisher, Conn, Vennesland & Westheimer (1953), however, have shown that dehydrogenating enzymes are highly stereo-specific, and it is therefore unlikely that the stearic acid dehydrogenase could affect all the hydrogen atoms on the $C_{(9)}$ and $C_{(10)}$ atoms. Furthermore, Fisher *et al.* (1953) found that whereas ethanol dehydrogenase could remove all the deuterium from deutero-diphosphopyridine nucleotide which had been prepared enzymically, it could remove only about half from deutero-diphosphopyridine nucleotide prepared chemically. The residual deuterium in the chemically reduced diphosphopyridine nucleotide was presumably in positions not accessible to the dehydrogenase. It therefore seems highly improbable that the stearic acid dehydrogenase of the goat could labilize tritium atoms not directly involved in the dehydrogenation.

Another possible explanation for our observations is that the tritium introduced on the $C_{(9)}$ and $C_{(10)}$ atoms of the stearic acid was more accessible to the dehydrogenase than the hydrogen already attached to these carbon atoms in the original elaidic acid. A consideration of the configurations of all the possible isomers of [9:10-$^3$H]stearic acid shows that, averaged over the whole molecular population, tritium and hydrogen are stereochemically equivalent, whether *cis*- or *trans*-oleic acid is used in the tritiation. This means that not more than half the tritium present could be removed during the formation of oleic acid unless the tritiation had been asymmetric; an asymmetric synthesis from the catalytic hydrogenation of non-optically active compounds has never been observed.

The only other possible explanation appears to be that the catalytically introduced tritium was not confined to the 8, 9, 10, 11 and 12 positions, as suggested above, but was distributed over more of the molecule than previous work would lead one to expect.

The fact that 1·68 times as much tritium was found in the 'methyl half' as in the 'carboxyl half' of the molecule might be accounted for by the finding of Moore (1919) that new double bonds can

form in both the 10:11- and 11:12-positions in one half of the molecule but only in the 8:9-positions in the other half. More tritium might thus enter the 'methyl half' of the molecule. Alternatively, the asymmetry of labelling of the oleic acid may be due to biological loss of tritium from the 'carboxyl half' of the molecule by, for example, $\beta$-oxidation and resynthesis. Neither of these mechanisms, however, would be expected to give rise to such a large difference between the tritium contents of the two halves of the molecule. Moreover, in this case, the ratio 1·68 is reliably known, there being good agreement between the values obtained from the duplicate samples. The most reasonable conclusion to be drawn from the work as a whole, therefore, is that the stearic acid produced by the catalytic addition of tritium to elaidic acid contains the isotope asymmetrically distributed over the molecule and not confined to the 9:10-positions.

## SUMMARY

1. Stearic acid labelled with tritium by catalytic addition, with a palladium-black catalyst, was fed to a goat, and the unsaturated fatty acids of the fat from two milk samples were separated by the lead-salt method. These were found to be radioactive.

2. From these unsaturated acids, after dilution with carrier oleic acid, dihydroxystearic acid was prepared and obtained in pure crystalline form. This was found to be radioactive, thus formally proving the biological conversion of stearic into oleic acid in the goat.

3. The dihydroxystearic acid from each sample was decomposed into pelargonic aldehyde and azelaic semialdehyde. Complete recovery of the tritium originally in the dihydroxystearic acid was obtained within the limits of experimental error. The conclusion is drawn that the tritium in the dihydroxystearic acid was stably bound. It was found further that 1·68 times as much tritium was contained in the pelargonic aldehyde as in the azelaic semialdehyde.

4. The two aldehydes were oxidized to the corresponding acids, thus labilizing the tritium originally attached to the $C_{(9)}$ and $C_{(10)}$ atoms. The loss of tritium was such that about 3 % of the total tritium in the dihydroxystearic acid molecule must have been attached to the $C_{(9)}$ atom and 12 % to the $C_{(10)}$ atom.

5. The significance of these findings is discussed in the light of possible artifacts, and the conclusion is drawn that the original stearic acid was asymmetrically labelled and that only about 28 % of the total tritium in the molecule was attached to the carbon atoms which were reduced.

This work was carried out while L. R. Reinius was the holder of a British Council Scholarship, which he acknowledges with thanks.

*Note added in proof.* We are indebted to Dr W. G. Duncombe for showing, by means of paper chromatography and autoradiography, that the labelled stearic acid, prepared from elaidic acid by the technique described, contains no chemical or radiochemical impurities.

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# Studies on the Origin of Milk Fat

## 2. THE SECRETION OF DIETARY LONG-CHAIN FATTY ACIDS IN MILK FAT BY RUMINANTS

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(*Received* 23 *July* 1955)

Early work, carried out chiefly by means of the arteriovenous technique, indicated that sufficient of the triglyceride fraction of blood is absorbed by the udder to account for all the fat of ruminant milk, and that no other fraction of blood fat is likely to be an important precursor (see reviews by Folley, 1940, 1949). However, it cannot be concluded that it is the fat absorbed from the blood which appears in the milk, for its metabolism in the udder is not known (Graham, Jones & Kay, 1936). This question can be examined in detail only by the use of isotopically-labelled fatty acids.

The origin of the short-chain fatty acids characteristic of the fat of ruminant milk has been explained in various ways, of which that proposed by Hilditch (1947) (see also Achaya & Hilditch, 1950) seemed at the time to be best supported by experimental evidence, though this is, admittedly, mostly indirect. According to this theory the short-chain acids are formed in the mammary gland by oxidative degradation of preformed oleoglycerides, beginning at the end of the carbon chain furthest from the carboxyl group. One molecule of oleic acid would thus give rise to one molecule of short-chain acid.

More recently, attention has been focused on the synthesis of fatty acids from smaller molecules, of which the most important is acetic acid, or a reactive derivative of acetic acid. That this occurs in the mammary gland has been shown by respiratory and isotope experiments on mammary slices *in vitro* (see Folley, 1954), and by isotope experiments on the intact goat (Popják, French & Folley, 1951) and on the isolated, perfused cow's udder (Cowie *et al.* 1951). It was found in these experiments that [$^{14}$C]acetate injected into the blood resulted in considerable radioactivity in all the acids of milk fat up to and including palmitic acid. Stearic and oleic acids, on the other hand, had a much lower specific activity, and it was thought that they might arise mainly from blood lipids.

This work with labelled acetate does not, however, give any indication of the quantitative importance of acetate as a precursor of milk fatty acids, and the possibility is by no means excluded that appreciable proportions of the lower acids arise by degradation of long-chain acids, as envisaged by Hilditch. In the experiments to be described, tritium-labelled stearic acid has been administered orally, either as free acid or as triglyceride, to lactating goats and a cow, and the rate of appearance and the distribution of radioactivity in the milk have been investigated. By this means the uptake of circulating long-chain acids by the mammary gland could be detected, Hilditch's theory could be tested, and an estimate could be made of the contribution of dietary stearic acid to milk fat.

Some of the results have already been communicated in preliminary form (Duncombe & Glascock, 1954; Glascock & Reinius, 1954).

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## EXPERIMENTAL

### *Analytical methods*

*Milk fat* was determined by the routine Gerber method.

*Isolation of milk fat for isotopic analysis.* About 100 ml. of each milk sample was centrifuged and the aqueous phase kept for isolation of other milk constituents. The cream was treated with boiling ethanol and ammonia and the lipids were extracted with a mixture of equal volumes of light petroleum (b.p. 40–60°) and ether (McClymont, 1951). The solution of lipids was dried over anhydrous $Na_2SO_4$, the solvent evaporated under reduced pressure, and the molten fat subjected to a vacuum of a few mm. Hg in a desiccator.

*Milk water* was obtained from the aqueous phase by freeze-drying about 0·1 ml. in a vacuum apparatus and collecting the sublimed water.

*Lactose* was prepared from the aqueous phase according to the method of Popják, Hunter & French (1953), and was washed with a solution in light petroleum (b.p. 40–60°) of tristearin and stearic acid in order to remove any traces of radioactive lipids, and then crystallized from methanol. After drying at 110° the lactose was characterized by formation of the osazone (m.p. 188–189°).

*Casein* was precipitated from the aqueous phase (diluted 1:4 with water) by adjusting the pH to 4·6 with acetic acid. The precipitate was filtered off, washed quickly with 0·02 M acetic acid, and dissolved by the addition of the minimum quantity of 0·1 N-NaOH. The solution was filtered and the casein again precipitated, dissolved, reprecipitated and

finally dried. It was then continuously extracted with ether to remove possible traces of radioactive lipids.

*Fractionation of milk fat.* The following fractions were obtained by the use of procedures similar to those described by Popják & Beeckmans (1950): glyceride fatty acids (non-volatile total, saturated and unsaturated; steam-volatile water-soluble and -insoluble) and phospholipid fatty acids (same fractions). Steam-volatile fractions were redistilled after addition of inactive carrier stearic acid, in order to avoid possible contamination by traces of radioactive long-chain acids.

*Glycerol* was isolated as the tribenzoate from the aqueous phase remaining after saponification of the glyceride fraction and extraction of the free fatty acids (Popják, Glascock & Folley, 1952), and was thoroughly washed with a carrier solution of stearic acid.

*Blood total solids and blood water* were obtained by freeze-drying small volumes.

*Determination of fat content of diet (Experiment IV).* Samples of each component of the diet were taken at intervals during the experiment and pooled. These were dried to constant weight at 100°, ground and extracted with ether in a Soxhlet apparatus. The ethereal extract was decolorized with activated charcoal, and the total pigment-free lipids were dried to constant weight after removal of the ether.

### *Radioactivity measurements*

Solid samples were subjected to combustion in a stream of oxygen (Glascock, 1952). The tritium content of the combustion water, and of blood- and milk-water samples, was determined by conversion into butane and measurement of the radioactivity in a gas counter (Glascock, 1951). All specific activities quoted are in counts/min./mg. of water as observed with our equipment.

### *Radioactive materials*

[$^3$H]Stearic acid was prepared from oleic or elaidic acid by hydrogenation with tritium gas (Glascock, 1954; Glascock & Reinius, 1956). Labelled triglycerides were prepared by transesterification of tristearin or olive-oil triglycerides with the labelled acid, possible traces of free fatty acid and phospholipid being removed by passage of an acetone solution of triglyceride through a magnesium oxide column (Bergström, Borgström & Rottenberg, 1952).

### *Animal experiments*

*Experiment I.* The animal was a cross-bred British Saanen goat weighing 73·5 kg., pregnant, and giving about 1·5 kg. of milk daily. The labelled material consisted of 11·7 mg. of [$^3$H]stearic acid (prepared from oleic acid) containing a total activity of $4{\cdot}05 \times 10^9$ counts/min. It was dissolved in 1 ml. of arachis oil and emulsified in 50 ml. of 5% (w/v) glucose solution (hot) together with 0·5 g. of glyceryl monostearate. The goat was injected with oxytocin and milked out, and the cooled emulsion was immediately administered quantitatively by mouth, followed by 50 ml. of similar but non-radioactive emulsion. Water, hay and stock cereal diet were provided *ad lib.*, and the goat was kept in an isolation pen and milked once daily for 15 days. Bedding and droppings were burnt daily. In this and subsequent experiments milk samples were treated in the manner already described.

*Experiment II.* In the preceding experiment the labelled stearic acid was synthesized from oleic acid. This is very difficult to obtain in a state of chemical purity, and impurities might give rise, on saturation with tritium-hydrogen, to labelled acids of chain length other than $C_{18}$. In the present experiment the starting material was elaidic acid, the crystalline *trans*-isomer of oleic acid, which can be obtained chemically pure. The labelled material administered orally to the goat was an emulsion containing 7·7 mg. of [$^3$H]stearic acid, prepared from elaidic acid, and having a total activity of $3{\cdot}94 \times 10^9$ counts/min.

This goat was a pedigree British Saanen weighing 61·1 kg. and giving 2–3 kg. of milk daily. After administration of the emulsion the goat was milked out at 2-hourly intervals for the first 10 hr., and twice daily afterwards for 14 days. Every 2 hr. during the first day 5 ml. blood samples were taken from the jugular vein and afterwards once daily.

*Experiment III.* This experiment was designed so that some estimate might be made of the quantitative contribution of dietary fat to milk fat. The labelled stearic acid was fed as tristearin, and the dietary fat was arranged to consist mostly of triglyceride by using a diet deficient in fat and augmented with tristearin and milk.

The animal was a pedigree British Saanen goat weighing 46·8 kg., and giving about 3 kg. of milk daily. The daily diet contained sugar-beet pulp and potato flakes (about 2 kg.); hay (about 450 g.); casein (about 100 g.); milk (about 2 kg.), and tristearin (about 75 g. as a 10% emulsion mixed with the milk). The exact amount of each component consumed was recorded daily and its fat content determined, so that the daily intake of fat could be calculated. During the 7-day experimental period the total consumption of fat was 1197 g., derived as follows: sugar-beet pulp and potato flakes, 22%; hay, 2%; milk, 32%; tristearin, 44%. Since milk fat consists largely of triglycerides (Bloor, 1943), it can be seen that at least 75% of the dietary fat must have been in this form. The goat was gradually accustomed to this diet during the 3 weeks preceding the experimental period.

The labelled material consisted of 2·72 g. of tristearin containing combined [$^3$H]stearic acid prepared from elaidic acid. The total activity was $1{\cdot}24 \times 10^9$ counts/min. This preparation was administered orally in an emulsion, and the goat was milked twice daily. Total faeces were collected daily and dried, and the tritium content was measured.

*Experiment IV.* A cow was used in this experiment, and here, also, the labelled fatty acid was fed as triglyceride. However, in order to overcome the objection that tristearin is not a common dietary constituent and might abnormally influence utilization of fat, a tracer dose of labelled olive-oil triglyceride was administered.

The cow was a pedigree Shorthorn in her fourth lactation, weighing 567 kg. and giving about 10 kg. of milk daily. The diet consisted of hay, concentrates and silage, each from the same stock throughout the experiment. The daily consumption of each of these was measured. Fat was determined in each component of the diet as already described and the daily intake of fat calculated.

The labelled material consisted of 4 g. of olive-oil triglycerides containing combined [$^3$H]stearic acid prepared from elaidic acid. The total activity present was $2{\cdot}25 \times 10^{10}$ counts/min. This oil was administered by mouth in an

emulsion immediately after milking, and the cow was afterwards milked at 4-hourly intervals for the first 40 hr. Normal twice-daily milkings were then resumed.

## RESULTS

*Experiment I.* The specific activity of the milk fat is shown plotted semi-logarithmically against time in Fig. 1 (*a*), the time for each point on the curve being the mid-point of the time during which the sample was secreted. It will be observed that milk having the highest specific activity was secreted during the first 24 hr. The specific activity then declined rapidly. The half-life was not constant, however, indicating that a number of processes with different time-constants are involved in the transport of the radioactivity from gut to milk. in the milk. It is of interest to note that both triglyceride and phospholipid long-chain acids of milk have about the same specific activity; they are evidently derived ultimately from the same pool.

An unexpected feature of the results was the comparatively high specific activity found in the unsaturated glyceride acids. It is well known that saturation and desaturation of fatty acids occur in the body. If, however, the stearic acid is labelled with tritium only at the $C_{(9)}$ and $C_{(10)}$ atoms, as expected from the method of synthesis, then biological desaturation would be expected to lead to the loss of approximately half the radioactivity, assuming that no isotopic fractionation had occurred. Further investigation of this problem, using samples obtained in Expt. II, has, in fact, shown

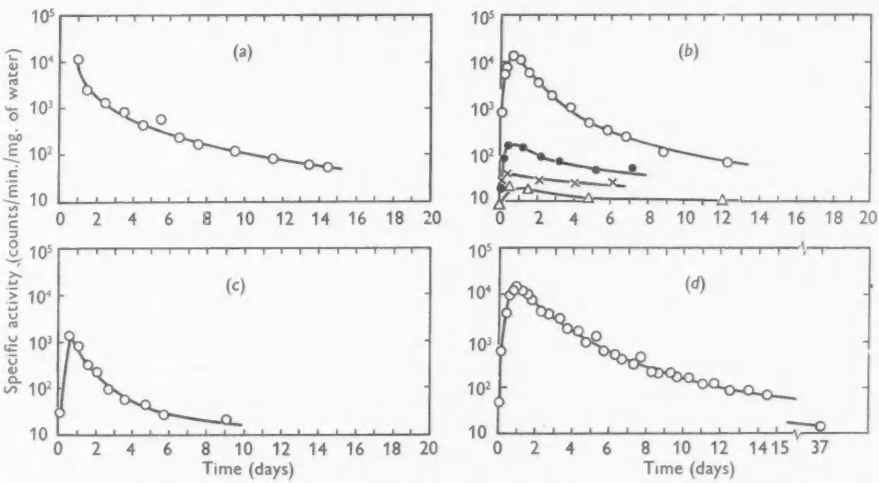


Fig. 1. Specific activity of milk and blood constituents of goats (*a*, *b* and *c*) and a cow (*d*) after ingestion of [$^3$H]stearic acid administered as free acid (*a* and *b*) or as triglyceride (*c* and *d*). ○, Milk fat; ●, blood solids; ×, blood water; △, milk water.

The cumulative secretion of activity in the milk fat, as a percentage of the dose administered, is shown in Fig. 2 (*a*). More than 17% of the dose was secreted in the first 24 hr., the total secretion being substantially complete at about 26% of the dose after 8 days.

Fractionation of the fat from the first day's milk sample (Table 1, column 2) shows that most of the activity resided in the long-chain fatty acids, though there was also measurable activity in the short-chain acids, and in glyceride glycerol, cholesterol and lactose. It thus seems probable that dietary long-chain fatty acids can be transported intact to the mammary gland and secreted that the stearic acid was not labelled as expected (Glascock & Reinius, 1954, 1956). In the oleic acid biologically derived from it only about 3% of the total tritium present resided on the $C_{(9)}$ atom and 12% on the $C_{(10)}$ atom. Furthermore, the activity in atoms $C_{(10)}$ to $C_{(18)}$ was 1·68 times that in atoms $C_{(1)}$ to $C_{(9)}$. The finding that the unsaturated acids in the present experiment have quite a high specific activity is thus consistent with the occurrence of biological desaturation of long-chain acids in the intact goat.

*Experiment II.* One of the objects of this experiment was to investigate more closely the early part of the milk-fat specific activity/time curve

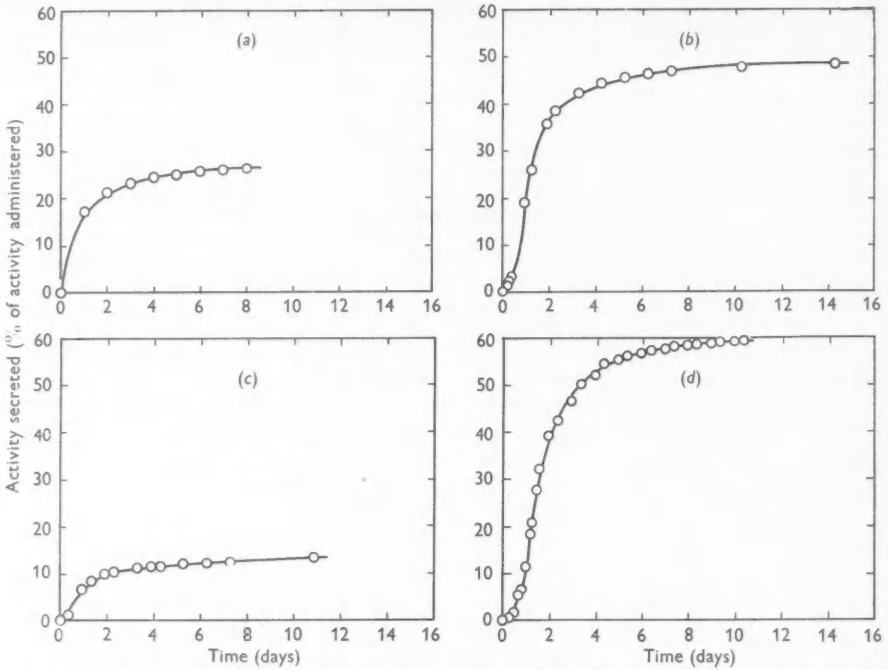


Fig. 2. Cumulative secretion of radioactivity in milk fat of goats (*a*, *b* and *c*) and a cow (*d*) after ingestion of [$^3$H]stearic acid administered as free acid (*a* and *b*) or as triglyceride (*c* and *d*).

Table 1. *Specific activities (counts/min./mg. of water) of milk constituents from goats and a cow after the oral administration of* [$^3$H]*stearic acid as free acid or as triglyceride*

For experimental details see text.

| Constituents | Expt. I | Expt. II | | Expt. IV |
|---|---|---|---|---|
| | | Sample 6 | Sample 7 | |
| Total lipids | 11 460 | 12 880 | 10 610 | 14 200 |
| Glyceride fatty acids: | | | | |
| Non-volatile total | 11 835 | — | — | — |
| Non-volatile saturated | 14 000 | 12 770 | 10 900 | 14 280 |
| Non-volatile unsaturated | 11 030 | 10 800 | 10 200 | 19 540 |
| Steam-volatile water-soluble | 80 | 34 | 68 | 24 |
| Steam-volatile water-insoluble | 206 | 196 | 450 | 198 |
| Glyceride glycerol | 346 | 144 | 316 | — |
| Phospholipid fatty acids: | | | | |
| Non-volatile total | 13 290 | — | — | — |
| Non-volatile saturated | — | — | — | 13 000 |
| Non-volatile unsaturated | — | — | — | 19 000 |
| Steam-volatile water-soluble | 201 | — | — | — |
| Steam-volatile water-insoluble | 411 | — | — | — |
| Cholesterol | 69 | — | 51 | — |
| Lactose | 150 | 39 | 31 | 38 |
| Casein | — | 111 | 171 | — |

after the ingestion of a tracer dose of labelled free stearic acid. As will be seen from Fig. 1 (*b*), activity appears quite rapidly in the milk fat, an appreciable level being found in the sample taken 4 hr. after dosing; the peak activity is reached after 23 hr. Thereafter, the pattern is very similar to that found in the previous experiment.

The cumulative secretion of activity (Fig. 2*b*) has a similar form to that of the previous experiment. The total secretion is considerably greater here, however, reaching 47 % after 8 days (cf. 26 % in Expt. I) and 49 % after 14 days. Appreciable activity was still being secreted after 14 days.

The pattern of distribution of activity in milk-fat fractions and other milk constituents (Table 1, columns 3 and 4) also confirms the previous results.

The peak activity of blood solids occurs at about the same time as that of milk fat (Fig. 1*b*). During the early part of the experiment at least it seems probable that nearly all the radioactivity associated with blood solids is due to long-chain fatty acids absorbed from the gut, and these results indicate that circulating lipids are taken up by the mammary gland and secreted in the milk very rapidly.

It will be seen from Fig. 1 (*b*) that the blood-water and milk-water curves do not coincide. This is unexpected because labelled water is known to equilibrate very rapidly with body water. Although this finding cannot at present be explained, it is not thought that it is due to analytical errors.

From these results it is also possible to calculate the percentage of administered activity circulating in blood solids or as body water at any time. Taking the blood volume of the goat as 6·2 % of its body weight and the total solids content of the blood as 20·5 g./100 ml. (Dukes, 1947), then the solids of the blood amount to about $7{\cdot}7 \times 10^5$ mg. Combustion of dry blood solids showed that 1 mg. gave 0·72 mg. of water. The highest measured specific activity of the blood solids was 156 counts/min./mg. of combustion water. The total activity circulating in blood solids at that time was therefore $8{\cdot}65 \times 10^7$ counts/min., corresponding to 2·2 % of the activity administered. Similarly, by taking body water to be 70 % of the body weight, and assuming that blood water is characteristic of all body water, it is calculated that when the blood water is at its peak specific activity, about 41 % of the total activity administered is present in the body in the chemical form of water. Because of the low specific activity of the body water, at least 41 % of the ingested activity is therefore lost as far as fatty acid metabolism is concerned. Of the 59 % remaining, more than 48 % is secreted in the milk fat, from which it is evident that the lactating mammary gland takes a large share of the labelled stearic acid available to the whole body.

*Experiment III.* In this experiment the labelled material was administered as triglyceride instead of free acid. The rate of appearance of activity in the milk fat is hardly changed, however, as may be seen by comparing Fig. 1 (*c*) with Fig. 1 (*a*) and (*b*), and the peak activity is again reached in less than 24 hr. after dosing. The total secretion of activity in milk fat is, however, considerably less than after ingestion of labelled stearic acid, the plateau level reaching only about 13 % of the ingested activity (Fig. 2*c*).

The controlled fat intake of the goat in this experiment enables an estimate to be made of the contribution of dietary glycerides to milk fat. During the first 7 days of the experiment 1197 g. of fat was ingested, and as an approximation it may be assumed that this amount of glyceride fat became labelled with the total radioactivity given in the dose. About 12·5 % of this activity was secreted in the milk during the first 7 days; thus 12·5 % (149 g.) of the dietary glyceride appeared during that time in the milk-fat of which the total output was 623 g. Thus 149/623, or 24 % of the milk fat, was derived from dietary glycerides. This is an upper limit for the contribution, the assumption being made that the dietary fat consisted entirely of glycerides. In fact, at least 75 % of the dietary fat is known to have been glycerides (see Experimental), so that the lower limit to the contribution becomes about 18 %. There are, of course, considerable uncertainties in such a calculation. In particular, the assumption is implicit that all long-chain fatty acids behave similarly with respect to absorption from the gut, transport and utilization by the mammary gland. If stearic acid does not behave quantitatively in the same way as other esterified long-chain acids then the calculation will not be valid.

It is of interest to note that this calculation does not depend on the assumption that all the labelled glyceride was absorbed from the gut. As a matter of interest measurements of total faecal dry matter and its specific activity were made and these showed that 33·9 % of the total activity ingested was excreted, so that only 66·1 % was absorbed. The figure of 12·5 % of the total ingested activity appearing in milk fat in 7 days (Fig. 2*c*) thus corresponds to 18·9 % of the absorbed activity.

*Experiment IV.* The rate of appearance of activity in the milk fat of a cow after oral administration of labelled triglyceride (Fig. 1*d*) is in general similar to the curves previously found for goats given free stearic acid or triglyceride (cf. Fig. 1*a–c*), and the maximum activity occurs at about the same time. Measurable activity was still present in the milk fat secreted after 37 days.

The cumulative secretion of activity was the highest found in any experiment, reaching 59 %

after 10 days (Fig. 2*d*). From the specific activity of blood water 2 days after dosing it was calculated (as in Expt. II) that about 40 % of the ingested activity was at that time in the chemical form of water. In this particular animal, therefore, nearly all the ingested activity could be accounted for either as milk fat or as water. Absorption must therefore have been virtually complete. This result is in contrast with that of Expt. III, which was carried out on the goat, in which 33·9 % of the ingested activity was excreted. This is possibly because the diet in that experiment contained a considerable amount of tristearin, which is an unphysiological ingredient.

In the first 10 days the fat intake of the cow was 2070 g. (ether-extractable fat, decolorized with charcoal), and the production of milk fat was 4525 g. Using the figure of 59 % of the dose appearing in the milk fat, a calculation similar to that employed in Expt. III shows that some 27·5 % of the milk fat was derived from dietary fat, compared with 18–24 % found for the goat. This is almost certainly an upper limit for the contribution, since it is assumed in the calculation that all the dietary fat becomes effectively labelled with the ingested, labelled triglyceride. If, as is possible, some of the dietary fat consists of waxes or other lipids not utilized by the mammary gland in the same way as glycerides, then the true contribution of dietary fat to milk fat would be less than that calculated.

Fractionation of the milk-fat sample of highest specific activity (Table 1, column 5) gave results generally in good agreement with those obtained from goats given labelled unesterified stearic acid, but differing in that the unsaturated long-chain acids of both triglycerides and phospholipids here have a considerably higher specific activity than the corresponding saturated acids. Whether this difference from the previous results is due to the chemical form of the ingested acid cannot at present be determined.

## DISCUSSION

In the experiments reported here the labelled materials were administered by mouth. A considerable degree of randomization and transformation of lipids takes place during digestion and absorption in laboratory animals, on which most work has been carried out. The administered radioactivity thus becomes distributed in the various fractions of the blood lipids. This leads to considerable difficulties in the interpretation of milk-fat radioactivities when a specific precursor is being sought, even if detailed fractionation of blood lipids is attempted. Nevertheless, it is clear from these results that some part of the milk fatty acids is derived from circulating lipids without extensive breakdown and resynthesis. The long-chain fatty acids of milk had a very much higher specific activity than the short-chain acids, and other fractions of the milk, including glyceride glycerol, cholesterol, lactose and casein, were found to have a comparatively low specific activity. Obviously, therefore, much of the milk long-chain acids must have arisen other than by synthesis from small molecules, and it seems certain that this is by direct transport of dietary fatty acids. The results suggest, however, that the contribution of dietary fat to milk fat may not be more than about 25 %. The original estimate of 70 % (Duncombe & Glascock, 1954) was based on a dietary fat content taken as the total ether-extractable matter, and is evidently considerably too high.

There are virtually no other figures available to enable a complete balance sheet of milk-fat production to be attempted. Results from non-isotopic techniques, such as measurement of arteriovenous differences, are difficult to interpret even qualitatively since the use to which absorbed fat is put by the mammary gland is unknown. In future developments of this work it is hoped to investigate the importance of the various fractions of blood lipids as precursors of milk fat. The quantitative importance of acetate and other short-chain acids absorbed from the rumen must also be studied before there is a possibility of obtaining a complete picture.

The question whether the short-chain fatty acids of milk arise by degradation of long-chain acids can now be answered with a fair degree of certainty. In all these experiments the short-chain acids have had a much smaller specific activity than the long-chain acids, and it is clear that not very much chain-shortening of the higher acids could have occurred. It should be noted that this conclusion is valid only if chain-shortening would give lower fatty acids still containing tritium. On Hilditch's theory, it is the oleic acid in glyceride combination that undergoes chain-shortening, starting at the methyl end of the molecule. As mentioned above, the oleic acid obtained biosynthetically in the present experiments was found to be labelled with tritium on atoms other than $C_{(9)}$ and $C_{(10)}$ (Glascock & Reinius, 1956), so that acids down to $C_{(10)}$ formed from it would almost certainly have a high specific activity, while shorter acids might also be appreciably active. The finding of only small specific activities in both water-soluble and -insoluble steam-volatile fatty acids suggests that these fractions did not arise primarily by degradation of oleic (or stearic) acid, and is consistent with the synthesis which is known to occur from circulating short-chain acids. This synthesis, contrary to the theory of Hilditch, may thus be the quantitatively

predominant process in the production of the characteristic short-chain acids of ruminant-milk fat.

Hilditch (1952) has more recently suggested that his analytical observations could be explained by assuming that oleic acid residues in glyceride combination might, instead of being degraded to short-chain acids, be replaced by short-chain acids derived from some other source. This would indeed help to reconcile the known facts with his observations, although other questions are raised, such as the fate of the oleic acid residues displaced from the glycerides. These might perhaps be oxidized, or re-incorporated into glycerides if more glycerol is made available by synthesis. Such possibilities might well be studied by isotopic techniques.

## SUMMARY

1. Tritium-labelled stearic acid was administered orally, either as free acid or as triglyceride, to lactating goats and to a cow, and the appearance of radioactivity in milk fat and its distribution in milk constituents were studied.

2. Measurable activity was found in the milk fat 4 hr. after administration of labelled fat, the maximum specific activity occurring within 24 hr. Activity was still detectable in milk fat secreted up to 37 days after administration.

3. Up to 59% of the ingested activity ultimately appeared in the milk fat.

4. High specific activities were found in the long-chain acids, both saturated and unsaturated, of milk glycerides and phospholipids. Much lower specific activities were found in the steam-volatile water-soluble and -insoluble fatty acids of milk fat, and in milk glyceride glycerol, lactose, cholesterol, casein and in body water.

5. The results suggest that the contribution of dietary fat to milk fat was not more than about 25%, and that the characteristic short-chain acids of the milk fat of ruminants do not arise mainly by degradation of long-chain acids.

We wish to record our indebtedness and thanks to the following: Dr S. J. Folley, F.R.S., and Mr A. S. Foot for making available the experimental animals; Dr R. E. Bowman of Parke, Davis and Co. for the gift of elaidic acid, and Unilever Ltd. for the gift of 6 kg. of tristearin; Dr S. J. Rowland for the milk fat determinations; Mr B. W. E. Peaple for assistance in many ways, including determinations of radioactivity; and Mr B. B. Brown for supervising the management of the cow in one experiment, and for taking many milk and blood samples. One of us (L.R.R.) was the holder of a British Council Scholarship during the course of this work.

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# The Separation and Determination of Microquantities of Lower Aliphatic Acids, including Fluoroacetic Acid*

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(*Received* 6 *May* 1955)

Although various methods of paper-chromatographic separation of fatty acids, using either their salts or the corresponding hydroxamic acids, have been described (Block, Durrum & Zweig, 1955), several problems still remain unsolved. Thus the quantitative separation of microquantities of formic and acetic acids by chromatography on paper has met so far with little success (Brown, 1950; Hall, 1950) and no method has yet been found for the separation and quantitative estimation of fluoroacetate, which in biological fluids is always accompanied by acetate. Fluoroacetate is a substance of great biochemical interest and is widely used as rodenticide. The analysis of foodstuffs, body fluids and tissues for this poison is therefore often desirable. Chromatographic separation of fluoride and fluoroacetate on a silicic acid column has been attempted by Ramsey & Clifford (1949), and the applicability to fluoroacetate of the lanthanum nitrate method for acetate has been reported by Hutchens & Kass (1949). However, these procedures do not permit the quantitative determination of microquantities of fluoroacetate, such as can be expected in biological material.

In the present investigation two problems have been studied: (*a*) the paper-chromatographic separation of formate and fluoroacetate from acetate and the other lower fatty acids; (*b*) the quantitative determination of microquantities of fatty acids, including fluoroacetate, from paper chromatograms.

After many unsuccessful trials with the sodium or ammonium salts of these acids our efforts were concentrated on the study of the corresponding hydroxamic acids. Appropriate conditions were found for the separation of formhydroxamic and fluoroacetohydroxamic acids from the hydroxamates of other lower fatty acids. In addition, a new colorimetric procedure for the quantitative determination of these derivatives has been found, with a lower limit of sensitivity of about 0·5 μg./ml. of final solution. The principles of the new method are described in the present paper. Application to body fluids and tissue extracts will be reported in a later communication.

* Presented at the Third International Congress of Biochemistry, Brussels, August 1955.

## MATERIALS AND METHODS

Benzohydroxamic acid, m.p. 131°, was prepared according to Hauser & Renfrow (1943). α-Naphthylamine was recrystallized twice from light petroleum (b.p. 60–80°). All solvents used were commercial products, purified by distillation.

*Preparation of hydroxamic acids.* A portion of the solution to be examined, representing about 1 mg. of organic acid, was made alkaline with ammonia, the solution evaporated to dryness and the residue extracted twice with 1 ml. of 0·4N-HCl in ethanol. Ethereal diazomethane was added until a persistent yellow colour was obtained, and after 10 min. standing the excess of reagent was destroyed by addition of a few drops of 0·4N-HCl in ethanol. Hydroxylamine reagent (equal vol. of 2N hydroxylamine sulphate and 3·5N-NaOH) (2 ml.) was added and the mixture left at room temp. for 15 min. The solution was then concentrated on a water bath to a definite volume and a portion, representing 5–50 μg. of organic acid, spotted on Whatman no. 1 paper.

*Paper-chromatographic separation of hydroxamic acids.* The spots were first developed with a basic solvent mixture (solvent B): 95% ethanol, containing 5% $NH_3$–pyridine–water (3:1:1, by vol.). The paper was then dried, turned through 90° and developed with an acidic mixture (solvent A): 95% ethanol–dioxan–water–acetic acid (60:20:19:1, by vol.).

The $R_F$ values given in the tables were determined in ascending chromatograms, which were developed for about 5 hr. (solvent front 12–15 cm. from the original spots).

*Qualitative determination and location of spots.* The procedure of Fink & Fink (1949) for the detection of hydroxamic acids was modified by the use of 1% $FeCl_3$ in 95% ethanol, containing 0·1% HCl. This reagent is preferable to the butanol solution, since drying takes place much faster and the staining is much clearer. The hydroxamic acids appeared as violet spots on a yellow background. This procedure was applied for the measurement of $R_F$ values. Also, in analytical runs, a sample of the substance to be determined was run at the side of the paper, which was cut off and stained with $FeCl_3$ for location purposes. After some experience, however, this becomes unnecessary.

*Colorimetric analysis of hydroxamates by conversion into nitrite.* For quantitative analysis of the hydroxamic acids after chromatographic separation an area of 2 × 2 cm., containing the unstained material, was extracted with 5 ml. of 5% sodium acetate solution at room temp. for 15 min. Then 0·5 ml. of 1% sulphanilic acid in 25% (v/v) aqueous acetic acid was added, followed by 5 drops of $I_2$ solution (1·3 g. of $I_2$ in 100 ml. of acetic acid) and the mixture left for 4 min. Excess of $I_2$ was destroyed with

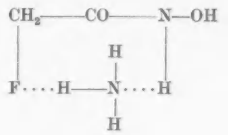
2-3 drops of 0·1 N-$Na_2S_2O_3$, then 2 drops of naphthylamine reagent (0·6% α-naphthylamine in 30% (v/v) acetic acid) and water to 6 ml. were added. The solution was decanted from the paper and read after 10 min. in a Klett–Summerson photoelectric colorimeter, using filter no. 52 (peak transmission 520 mμ.).

## RESULTS

### *Separation of acetate and fluoroacetate*

The sodium or ammonium salt of fluoroacetic acid is not suitable for chromatography, since none of the common indicators can be used for spot detection. With the corresponding hydroxamic acid no separation from acetohydroxamic acid was obtained with the usual acid solvent mixtures (Thompson, 1951). When the solvent contained pyridine, a higher $R_F$ value was observed for fluoroacetohydroxamic acid than for the acetate derivative, but the former could not be separated from propionate and butyrate.

It was then thought that the greater acidity of the fluoro compound might permit separation from the weaker aliphatic hydroxamic acids in a suitable pH range, where only the fluoro compound undergoes dissociation. However, the results in Table 1 show that within the pH range 5–11 separation could not be effected. When, however, instead of the various possible factors was made and the following results were obtained: (*a*) ammonia in ethanol, without further components, produces separation of fluoroacetate and acetate. However, considerable trailing ensues and makes this simple solvent unsuitable for quantitative analysis. On addition of pyridine, the spots become concentrated. (*b*) When the ammonia molecule is gradually substituted by alkyl groups, the difference in $R_F$ value between acetate and its fluorine derivative decreases more and more and disappears completely for trimethylamine (see Table 1). The difference between formohydroxamic and acetohydroxamic acids is of the same order in ammonia, mono- and di-methylamine, but is much less in trimethylamine. It can thus be concluded that at least one hydrogen atom must be present in the base. With fluoroacetohydroxamic acid it appears possible that intramolecular hydrogen bonding of the type (I) is responsible for the very low migrating



(I)

Table 1. *$R_F$ values of normal aliphatic hydroxamic acids in various solvent mixtures*

The solvents were composed as follows: 1, 95% ethanol–pyridine–0·01 M phosphate buffer (60:20:20, by vol.), apparent pH of mixture 5·6; 2, as 1, apparent pH 8·0; 3, as 1, but with 0·01 M borate buffer, apparent pH 9·5; A, 95% ethanol–dioxan–water–acetic acid (60:20:19:1, by vol.); 4, dioxan–acetic acid (4:1, by vol.); 5, 95% ethanol–pyridine–water (3:1:1, by vol.) with added base as indicated, calculated as the gaseous form.

| | Solvent | Formate | Fluoroacetate | Acetate | Propionate | Butyrate | Valerate |
|---|---|---|---|---|---|---|---|
| 1 | | 0·78 | 0·84 | 0·84 | 0·88 | 0·90 | 0·92 |
| 2 | | 0·81 | 0·83 | 0·84 | 0·89 | 0·92 | 0·95 |
| 3 | | 0·75 | 0·81 | 0·81 | 0·85 | 0·90 | 0·92 |
| A | | 0·71 | 0·79 | 0·79 | 0·87 | 0·90 | 0·92 |
| 4 | | 0·31 | 0·70 | 0·54 | 0·77 | 0·85 | 0·86 |
| 5 | +1% $NH_3$ | 0·27 | 0·53 | 0·67 | 0·78 | 0·86 | 0·89 |
| | +2% $NH_3$ | 0·26 | 0·50 | 0·65 | 0·77 | 0·84 | 0·85 |
| | +3% $NH_3$ | 0·25 | 0·45 | 0·64 | 0·74 | 0·83 | 0·85 |
| | +5% $NH_3$* | 0·25 | 0·37 | 0·59 | 0·68 | 0·76 | 0·84 |
| | +1% $CH_3NH_2$ | 0·20 | 0·47 | 0·54 | 0·62 | 0·68 | (diffuse) |
| | +1% $(CH_3)_2NH$ | 0·27 | 0·55 | 0·61 | 0·68 | 0·72 | 0·73 |
| | +1% $(CH_3)_3N$ | 0·70 | 0·75 | 0·76 | 0·80 | 0·83 | 0·85 |

* Solvent B (see Methods).

buffer ammonia was used to produce an alkaline pH, a lower $R_F$ value was observed for fluoroacetate than for acetate. Following this lead the solvent mixture B was developed (see under Methods), which produced satisfactory separation of formate, fluoroacetate and acetate or higher fatty acids.

Since the curious effect of ammonia cannot be due to the pH of the solution, a thorough study of power of this molecule. However, no explanation for the anomalous behaviour of formohydroxamic acid is available at present. When log $(1/R_F - 1)$ was plotted as function of $n$, the number of carbon atoms attached to the carboxyl group (Martin, 1950), the value for $n = 0$ fell above the straight line characteristic for the other fatty acids when ammonia, mono- or di-methylamine was used for chromatography.

*Quantitative determination of hydroxamic acids after paper-chromatographic separation*

Staining of the hydroxamic acids with ferric chloride in ethanol sets a lower limit of detectability of about 5 $\mu$g./spot. Such small quantities are, however, insufficient for extraction and colorimetric determination; e.g. in a Klett–Summerson colorimeter at least 0·3 $\mu$mole in a final volume of 5 ml. is required. In the search for a more sensitive method, attention was directed towards Blom's (1926) observation that hydroxylamine can be determined in fractions of a microgram by oxidation to nitrite, using iodine. In the present case this would require hydrolysis of the hydroxamic acid before the determination of the hydroxylamine (Feigl, 1954). We have, however, found that iodine oxidation is applicable to the hydroxamates themselves. The liberated nitrite is used to form a red azo dye. This reaction was first studied with crystalline benzohydroxamic acid, which gave identical calibration curves, whether tested in aqueous solution or after spotting on paper and subsequent extraction with 5% sodium acetate (see Fig. 1). Likewise, sodium acetate or fluoroacetate, when converted into the corresponding hydroxamic acids by the procedure described under Methods, and then spotted on paper and chromatographed with solvent A, gave well-reproducible, linear calibration curves, indicating that this method lends itself to accurate quantitative analysis. As is seen in Fig. 1, the lower limit of this method is 0·5 $\mu$g./ml. of final solution.

Since the chromatographic separation requires application of solvent B (see above), it was necessary to check whether the large excess of hydroxylamine, used in the preparation of hydroxamates, becomes quantitatively separated from the hydroxamates. Fortunately, it was found that the base migrates with the solvent front. However, an unforeseen difficulty arose. When filter paper was soaked in solvent B and then air-dried it showed a colorimetric blank value of considerable magnitude, which made the determination of microquantities of hydroxamic acids illusory. The blank value increased with increasing exposure of the paper to air. Apparently, autoxidation of the basic components of the solvent formed substances which were oxidized by iodine to nitrite and thus produced the red azo dye from sulphanilic acid and $\alpha$-naphthylamine. However, when the paper was first soaked in solvent B, then dried and chromatographed with solvent A, containing acetic acid, the contaminating material was removed to the solvent front and the pure hydroxamates remained on the spots. Therefore, application of the iodine method to the chromatograms of hydroxamic acids requires two-dimensional chromatography, using first solvent B for separation and subsequently solvent A for purification.

With this method at hand, analyses were carried out with aqueous solutions (and with serum), containing 50 $\mu$g. each of formate, acetate and fluoroacetate per ml. After conversion into the hydroxamic acids, as described under Methods, one-tenth of the total volume was spotted on paper and submitted to two-dimensional chromatography. The final spots were located by the use of indicator samples run at the side of the paper and stained with ferric chloride. After elution and iodine oxidation, the red azo dye was formed and determined colorimetrically. The amounts, obtained by comparison with the calibration curves, were 4·7, 4·9 and 4·6 $\mu$g. respectively.

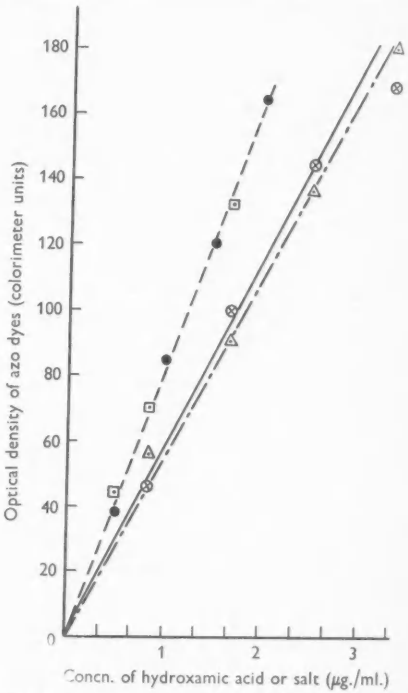



Fig. 1. Calibration curve for the colorimetric determination of hydroxamic acids by the nitrite reaction. ●, Aqueous solution of benzohydroxamic acid; ⊡, benzohydroxamic acid eluted from paper; ⊗, fluoroacetohydroxamic acid eluted after two-dimensional chromatography in solvents B and A. Concentration expressed as sodium fluoroacetate. Note the deviation from linearity with the highest value. △, Acetohydroxamic acid after two-dimensional chromatography, concentration expressed as sodium acetate.

Analysis of commercial preparations of fluoroacetate usually revealed the presence of a second substance of lower $R_F$ value. This was identified as chloroacetate, which results probably from the synthetic method used. As shown in Table 2, chloro- and bromo-acetohydroxamic acids have practically identical $R_F$ values and are easily separated from fluoroacetate.

The reaction between iodine and hydroxylamine is usually described by the following equation (Feigl, 1954):

$$NH_2.OH + 2I_2 + H_2O \rightarrow HNO_2 + 4HI.$$

Accordingly, each mole of hydroxamic acid should consume 4 equivalents of iodine. Direct titrations of aqueous solutions of benzohydroxamic acid with iodine in acetic acid consumed less than half of the 'theoretical' amount. However, a similar result was obtained with crystalline hydroxylamine hydrochloride itself. The reaction mechanism is therefore still obscure. However, in a great number of analyses the new procedure has given reproducible results. Application of the method to the analysis of urine and tissue extracts will be described in a subsequent paper.

Table 2. *$R_F$ values of halogeno-acetohydroxamic acids*

For solvent 5, see Table 1; 6, 95% ethanol–pyridine (4:1, by vol.) with ammonia; 7, 95% ethanol with 1% of gaseous HCl.

| Solvent | | Acetate | Fluoroacetate | Chloroacetate | Bromoacetate |
|---|---|---|---|---|---|
| 5 | +1% $NH_3$ | 0·67 | 0·53 | 0·32 | 0·30 |
| | +2% $NH_3$ | 0·65 | 0·50 | 0·26 | 0·26 |
| | +3% $NH_3$ | 0·64 | 0·45 | 0·24 | 0·23 |
| 6 | +0·5% $NH_3$ | 0·51 | 0·28 | ~0 | ~0 |
| | +1·5% $NH_3$ | 0·43 | 0·20 | ~0 | ~0 |
| 7 | | 0·80 | 0·75 | 0·31 | 0·31 |

## DISCUSSION

The present experiments show that the conditions for separation of formate and fluoroacetate from the other simple aliphatic hydroxamates are similar. Thus the method developed permits the simultaneous determination of formate and acetate, which so far met only with little success (Brown, 1950; Hall, 1950), and of acetate and fluoroacetate, which until now have resisted separation.

A new sensitive method for the quantitative determination of microquantities of hydroxamic acids has been found in the direct oxidation of them by iodine to nitrite, which then diazotizes sulphanilic acid to form an azo dyestuff with α-naphthylamine. This procedure is admirably suited for paper chromatography, since the excess of hydroxylamine is automatically separated from the hydroxamic acids.

In the nitrite method the nitrogen of the hydroxamates is the specific component which serves for colorimetric determination. Since filter paper does not contain any nitrogenous material it does not interfere with the procedure used. The advantage of this method becomes clear in the light of our efforts to determine hydroxamic acids by other means. For example, these derivatives, like hydroxylamine itself, reduce ammoniacal silver solutions, and under suitable conditions the reaction lends itself to a quantitative method for the determination of crystalline benzohydroxamic acid. However, since water or organic solvents extract from filter paper a considerable amount of reducing material, a high blank, varying with temperature and time of extraction, is obtained, which makes the silver method unsuitable for paper chromatograms.

It has been observed that solvent B produces on paper substances which are converted by iodine into nitrite. Analysis of this phenomenon revealed that both ammonia and pyridine are responsible for this effect. Apparently the cellulose surface has a catalytic action on the autoxidation of these bases, which are presumably transformed into amine oxides or related products.

## SUMMARY

1. Fluoroacetate can be separated from formate, acetate and other straight-chain fatty acids by paper chromatography of the hydroxamates in ammoniacal ethanol–pyridine–water.

2. The specific effect of ammonia is shared by mono- and di-methylamines, but is absent from trimethylamine. This may indicate the formation of an intramolecular hydrogen bond between an NH-group and fluorine.

3. Hydroxamic acids can be determined quantitatively by direct iodine oxidation to nitrite, which is used to form a red azo dye. The method has a lower limit of 0·5 μg./ml. of final solution.

4. The new procedure involves two-dimensional paper chromatography of the hydroxamic acids, extraction, and formation of the dye.

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# The Oxidation of Tryptophan and Some Related Compounds with Persulphate


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(*Received* 30 *June* 1955)

The oxidation of tryptophan and some related compounds with persulphate has been studied in the light of recent knowledge of the metabolism of this amino acid (cf. Dalgliesh, 1951). The compounds related to tryptophan which have been oxidized include kynurenine, 3-indolylacetic acid and indole, and the oxidation of anthranilic acid has been re-examined. A number of chemical oxidations of tryptophan have been reported, and usually they involve either attack on the side chain or fission of the pyrrole ring. With ferric chloride, for example, 3-indolylaldehyde is formed (Hopkins & Cole, 1903; Ellinger, 1906), and ozonolysis yields *N'*-formylkynurenine (Witkop & Graser, 1944; Knox & Mehler, 1950). Peracetic acid, however, hydroxylates L-tryptophan in the 2-position to yield β-3-oxindolylalanine (Witkop, 1947); Dalgliesh (1954) has shown that with the ascorbic acid-hydroxylating system of Udenfriend, Clark, Axelrod & Brodie (1954) and Brodie, Axelrod, Shore & Udenfriend (1954), 5- and probably also 7-hydroxytryptophan are formed. In the present work the action of alkaline persulphate on DL-tryptophan, under conditions similar to those previously described for the persulphate oxidation of aromatic amines (Boyland, Manson & Sims, 1953; Boyland & Sims, 1954), has led to the formation of a number of acid-labile and other products (see Fig. 1), some of which have either been isolated or identified by means of paper chromatography.

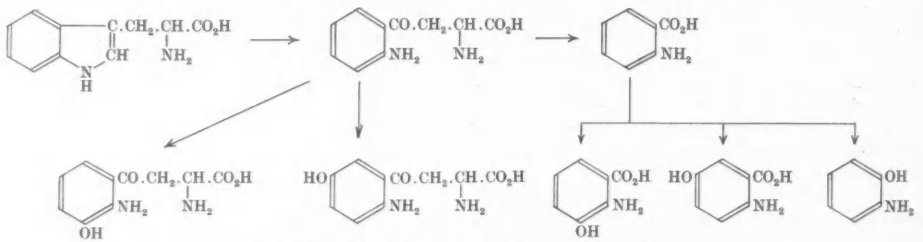

Fig. 1. Oxidation of DL-tryptophan with persulphate.

## EXPERIMENTAL

Melting points are uncorrected.

*Paper chromatography.* Descending chromatograms on Whatman no. 1 chromatography paper were developed with the organic phase of *n*-butanol–acetic acid–water (4:1:5, by vol.) (Partridge, 1946). The oxidation products were characterized on the papers by examination under a Hanovia Chromatolite ultraviolet lamp, and by spraying the papers (*a*) with 5% *p*-dimethylaminobenzaldehyde in 10% aqueous ethanol containing 2·5% HCl (Ehrlich's reagent), (*b*) with 0·4% ninhydrin in *n*-butanol saturated with water (the colours produced were sometimes slow in appearing, so that the chromatograms were examined 2 hr.

Table 1. *The colour reactions given by the acid-labile persulphate oxidation products after chromatography*

Methods are given in the Experimental section.

| Probable identity | $R_F$ | Fluorescence | Colour reactions: Ehrlich's reagent: Immediate | Ehrlich's reagent: After 2 hr. | Ninhydrin | $HCl + NaNO_2$ | $HCl + NaNO_2$ hexylresorcinol |
|---|---|---|---|---|---|---|---|
| *o*-Aminophenyl sulphate | 0·32 | — | Yellow | Yellow | — | Yellow | Scarlet |
| Sulphuric ester of 3-hydroxyanthranilic acid | 0·26 | Blue | Yellow | Pink | — | — | Yellow |
| Sulphuric ester of 5-hydroxyanthranilic acid* | 0·13 | Blue | Orange | Orange | — | — | Yellow |
| Sulphuric ester of 3-hydroxy-DL-kynurenine* | 0·04 | Blue-green | — | Pink | Reddish purple† | Yellow | Pink |
| Sulphuric ester of 5-hydroxy-DL-kynurenine* | 0·02 | Blue-green | Orange-yellow | Orange-yellow | Purple† | Yellow | Pink |
| Indoxyl sulphate | 0·25 | Violet | Brown | Blue† | — | Grey | Yellow, then blue |

* These esters could not be compared directly with the authentic compounds and their structures are inferred from the nature of their hydrolysis products.
† After 24 hr.

Table 2. *The colour reactions given by the persulphate oxidation products of tryptophan after acid hydrolysis and chromatography*

Methods are given in the Experimental section.

| Product | $R_F$ | Fluorescence | Colour reactions: Ehrlich's reagent | Ninhydrin: After 2 hr. | Ninhydrin: After 24 hr. | $HCl + NaNO_2$ | $HCl + NaNO_2$ hexylresorcinol |
|---|---|---|---|---|---|---|---|
| *o*-Aminophenol | 0·66 | — | Yellow | — | — | Brown | Crimson |
| 3-Hydroxyanthranilic acid | 0·80 | Blue | Yellow, turning pink | — | — | Greenish | Reddish brown |
| 5-Hydroxyanthranilic acid | 0·52 | Light blue | Orange-yellow | — | — | — | Scarlet |
| 3-Hydroxy-DL-kynurenine | 0·20 | Green | Pink | Pale yellow | Brown-purple | Brown | Reddish brown |
| 5-Hydroxy-DL-kynurenine | 0·10 | Yellowish green | Orange-pink | Pale yellow | Grey-purple | — | Pink |
| DL-Kynurenine | 0·23* 0·27 | Light blue | Orange | Pale yellow | Purple | — | Yellow |
| Anthranilic acid | 0·85 | Violet | Deep yellow | — | — | — | Orange-yellow |

* Resolved into D- and L-forms.

and again 24 hr. after spraying), and (*c*) with 2·5% aqueous $NaNO_2$ followed by N-HCl, noting any colour produced, and finally spraying with 0·5% hexylresorcinol in 2N-NaOH (cf. Booth, Boyland & Manson, 1955). Where possible the oxidation products were compared directly on the paper chromatograms with the reference compounds described below. The properties of the acid-labile compounds are listed in Table 1 and those of the phenols derived from them by acid hydrolysis in Table 2.

*Reference compounds.* DL-Kynurenine, anthranilic acid, 3-hydroxyanthranilic acid and *o*-aminophenol were obtained from commercial sources, and 5-hydroxyanthranilic acid was prepared by the method of Limpricht (1891). *o*-Aminophenyl sulphate was prepared from *o*-nitrophenol as described by Burkhardt & Wood (1929) and the sulphuric ester of 3-hydroxyanthranilic acid by the method of Boyland & Sims (1954). The latter ester, which was purified by several precipitations with 2N-HCl from its solutions in saturated aqueous $NaHCO_3$, formed one spot on paper chromatograms and yielded only 3-hydroxyanthranilic acid and inorganic sulphate after hydrolysis with hot 2N-HCl. 3- and 5-Hydroxy-DL-kynurenine were the gift of Professor A. Butenandt. These compounds had been prepared by the methods of Butenandt & Hellmann (1950) and Butenandt, Schulz & Hanser (1953) respectively. The *p*-toluidine salt of indoxyl sulphate obtained from Dr S. J. Holt had been prepared from the naturally occurring ester.

*Persulphate oxidations.* DL-Tryptophan (20 g.), DL-kynurenine (2·5 g.) or 3-indolylacetic acid (10 g.) was dissolved in aqueous 2N-KOH (20% excess of the theoretical

amount) and treated with aqueous $K_2S_2O_8$ (10% excess of the theoretical amount) added dropwise at room temp. over 6 hr. with continuous stirring. Some experiments were carried out with ice cooling, but this had no effect on the course of the reaction. Indole (10 g.) in 50% (v/v) aqueous acetone containing 2N-KOH (20% in excess of the theoretical amount) was similarly treated. The mixtures were kept overnight, evaporated to about 100 ml. under reduced pressure and made just acid with 10N-$H_2SO_4$. The dark flocculent precipitates which usually separated were filtered off and discarded. The filtrates were washed continuously with ether for some hours, and the aqueous layers were made alkaline with 2N-KOH and evaporated to dryness under reduced pressure; the residues were extracted several times with boiling methanol. The ethereal and methanolic extracts were treated as described below.

*Oxidation of tryptophan.* (*a*) *Ethereal extract.* A 2 ml. portion of the extract was evaporated and the residue was mixed with a few drops of 2N-HCl and a little magnesium powder was added. The mixture was filtered, and to the filtrate was added 75% $H_2SO_4$ (3 ml.) and a little chromotropic acid. The mixture was heated to 60°, and a violet-pink colour appeared, indicating the presence of formic acid in the ethereal extract (cf. Feigl, 1954).

The rest of the ethereal extract was evaporated to dryness, the residue was dissolved in saturated aqueous $NaHCO_3$ (2 ml.) and the solution was washed with ether (2 × 10 ml.). The aqueous layer was acidified with 2N-$H_2SO_4$ and extracted with ether. The ether was removed and the residue was recrystallized several times from ethanol to yield anthranilic acid (28 mg.), m.p. and mixed m.p. 142–144°. The identity of the acid was confirmed by paper chromatography.

(*b*) *The methanolic extract.* This was evaporated to dryness under reduced pressure to yield a brown gum which set to a brown resin. Examination of this resin on paper chromatograms showed the presence of unchanged DL-tryptophan together with a large amount of DL-kynurenine and small amounts of the products (except indoxyl sulphate) whose properties are listed in Table 1. When a little of the resin was heated to 100° with a few drops of 2N-HCl and the mixture examined on paper chromatograms, the phenols listed in Table 2 were detected. Among the products of the reaction detected on paper chromatograms which could not be identified was one forming spots ($R_F$ 0·04) which were non-fluorescent, but gave a purple colour with ninhydrin. When the resin was treated with hot 2N-HCl this substance could no longer be detected, but a second non-fluorescent substance of $R_F$ 0·16 was present, which gave a purple colour with ninhydrin.

A column 4 cm. in diameter and 20 cm. long was packed with Hyflo Super-Cel (Johns-Manville Co. Ltd., London, S.W.1) containing 25% (w/v) of water as a stationary phase, using a Martin packer (Randall & Martin, 1949). A little of the resin was dissolved in water (1 ml.) and added to a layer (2 cm.) of dry 'Super-Cel' which had been packed on the top of the column. The moving phase [amyl alcohol (British Standard 696)–*cyclo*hexane, 1:3, by vol.] was added and a pressure of 100 mm. Hg applied to the top of the column. The eluate was collected in 10 ml. fractions and these were examined in ultraviolet light. The fractions comprising 110–160 ml. of the eluate were mixed and concentrated, when DL-kynurenine, m.p. and mixed m.p. 205–210° (decomp.), was deposited as a yellow solid. (Found: N, 13·1. Calc. for $C_{10}H_{12}O_3N_2$: N, 13·5%.) The compound was indistinguishable from authentic DL-kynurenine on paper chromatograms.

*Oxidation of* DL-*kynurenine.* (*a*) *The ethereal extract.* Paper chromatography showed the presence of a compound with the same properties as anthranilic acid.

(*b*) *The methanolic extract.* This was evaporated to dryness under reduced pressure to leave a yellow solid. Examination of the solid on paper chromatograms revealed the presence of the products (except indoxyl sulphate) listed in Table 1. When the solid was heated to 100° for 15 min. with 2N-HCl these products could no longer be detected on paper chromatograms, but spots characteristic of the phenols listed in Table 2 were now present.

The solid was dissolved in water (100 ml.) and passed through a column 2 cm. in diameter and 15 cm. long of the acid form of the ion-exchange resin, Zeo-Karb 225. A further 100 ml. of water was passed through the column and the combined eluates were evaporated to dryness under reduced pressure. The solid residue was dissolved in the minimum of water and transferred on to a Hyflo Super-Cel column prepared as before. An equilibrated moving phase of amyl alcohol (B.S. 696)–light petroleum (b.p. 80–100°) (4:1, by vol.) was passed through the column, and the eluted fractions (*a*) 0–100 ml., (*b*) 200–300 ml. and (*c*) 300–400 ml. were collected separately and evaporated to dryness.

Fraction (*a*) formed a colourless powder which was shown by paper chromatography to contain a compound with the same properties as the sulphuric ester of 3-hydroxyanthranilic acid, together with small amounts of *o*-aminophenyl sulphate and a substance identical with one obtained in the anthranilic acid oxidation (see below).

Fraction (*b*) was dissolved in hot *n*-octanol (5 ml.) and allowed to crystallize in a vacuum desiccator; a small amount of pale-yellow needles separated, which were considered to be the sulphuric ester of 3-hydroxy-DL-kynurenine since the compound yielded only one spot on paper chromatograms and, after hydrolysis with hot 2N-HCl or with Taka diastase, only 3-hydroxy-DL-kynurenine could be detected on paper chromatograms. Inorganic sulphate was present in the hydrolysed solution.

Fraction (*c*) was a mixture which formed two spots on paper chromatograms, one of which was identical with that of the compound just described. When the mixture was hydrolysed as before, the hydrolysis product yielded two spots on paper chromatograms, identical with those formed by authentic 3- and 5-hydroxy-DL-kynurenine. It seems likely therefore that the second component of this fraction is the sulphuric ester of 5-hydroxy-DL-kynurenine.

The ion-exchange resin was treated with 2N-HCl (250 ml.) and the eluate was evaporated to dryness under reduced pressure. The solid was taken up in a little water and transferred to a column of Hyflo Super-Cel similar to that described above, but with a moving phase consisting of amyl alcohol–light petroleum (b.p. 80–100°) (1:9, by vol.). The eluted fraction 50–120 ml. yielded anthranilic acid, m.p. and mixed m.p. 142–143°. The eluted fraction 350–450 ml. yielded 3-hydroxyanthranilic acid, decomp. 210–240°. (Found: N, 9·3. Calc. for $C_7H_7O_3N$: N, 9·15%.) This compound formed one spot on a paper chromatogram identical with that of authentic 3-hydroxyanthranilic acid.

*Oxidation of* 3-*indolylacetic acid.* The ethereal extract was shown by paper chromatography to contain anthranilic acid.

The methanolic extract was examined by paper chromatography. A number of substances were present, but the only ones recognized had properties identical with the acid-labile compounds which were also obtained in the anthranilic acid oxidation described below.

*Oxidation of indole.* The ethereal extract was shown by paper chromatography to contain a substance indistinguishable from anthranilic acid.

The methanolic extract was evaporated to small volume under reduced pressure and allowed to crystallize. Potassium indoxyl sulphate (3·8 g.) separated; from aqueous ethanol it formed light brown plates. (Found: C, 38·5; H, 2·8; N, 5·3; S, 13·0. Calc. for $C_8H_6O_4NSK$: C, 38·2; H, 2·4; N, 5·6; S, 12·75%.) The *p*-toluidine salt separated from water in pale-blue plates. (Found: N, 8·9. Calc. for $C_{15}H_{16}O_4N_2S$: N, 8·75%.) The infrared spectrum of the *p*-toluidine salt was identical with that of the *p*-toluidine salt of the naturally occurring indoxyl sulphate.

The mother liquors were examined by paper chromatography and contained, in addition to indoxyl sulphate, the acid-labile products which were also formed in the anthranilic acid oxidation.

*Oxidation of anthranilic acid.* The oxidation of anthranilic acid with persulphate already described (Boyland & Sims, 1954) was re-examined and the mixture of acid-labile products which was isolated was examined by paper chromatography. The principal component of the mixture was the sulphuric ester of 3-hydroxyanthranilic acid, but a spot with properties identical with those of *o*-aminophenyl sulphate was also present. A third substance which formed spots on the chromatograms of the unhydrolysed mixture could no longer be detected by this means after the mixture had been heated with 2N-HCl for 15 min. These chromatograms now showed spots which could not be distinguished from authentic 3- and 5-hydroxyanthranilic acid and *o*-aminophenol respectively. It is considered likely, therefore, that the unknown acid-labile substance is the sulphuric ester of 5-hydroxyanthranilic acid.

The mixture of esters (500 mg.) was heated to 100° with 2N-HCl for 15 min. and the solution was treated with a small excess of solid $NaHCO_3$ and extracted with ether. Evaporation of the ether afforded *o*-aminophenol (10 mg.), which, after crystallization from water, had m.p. and mixed m.p. 173–174°.

## DISCUSSION

Alkaline persulphate appears to attack tryptophan at the 2:3-bond of the pyrrole ring. The first product of this reaction may be the disulphuric ester of 2:3-dihydro-2:3-dihydroxytryptophan, which would yield the free dihydroxy compound on acid hydrolysis. It is possible that the ester and the free hydroxy compounds are the substances with $R_F$ values 0·04 and 0·16 respectively, which were detected in the tryptophan oxidation. The substance of $R_F$ 0·16 is not β-3-oxindolylalanine, neither is it one of the hydroxytryptophans formed in the ascorbic acid oxidation system of Brodie *et al.* (1954) and of Udenfriend *et al.* (1954). 2:3-Dihydro-2:3-dihydroxytryptophan, it has been suggested (Knox & Mehler, 1950; Dalgliesh, Knox & Neuberger, 1951), is a possible intermediate in tryptophan metabolism.

Fission of the 2:3-bond of tryptophan would lead initially to the formation of *N'*-formylkynurenine. This has not been detected, but both formic acid and kynurenine are present in the reaction mixture. Only small amounts of the sulphuric esters derived from the further oxidation of tryptophan were detected, possibly because *N'*-formylkynurenine is only slowly hydrolysed during the reaction; acetyl derivatives of aromatic amines do not react with persulphate as do the parent amines (Boyland & Sims, 1954).

The sulphuric ester of 3-hydroxy-DL-kynurenine gives similar colours on paper chromatograms to those given by a substance which Dalgliesh (1952) detected in the urine of rats fed with supplementary tryptophan. That the substance was the sulphuric ester of 3-hydroxy-L-kynurenine was adduced by Dalgliesh from a consideration of some of its physical properties, together with the formation of 3-hydroxy-L-kynurenine and inorganic sulphate on acid hydrolysis.

In the oxidations of DL-kynurenine and anthranilic acid the substitution of sulphuric ester groups in positions both *ortho* and *para* to the amino groups is unexpected, in view of the fact that *para* substitution has not been detected in the persulphate oxidation of other aromatic amines (Boyland *et al.* 1953; Boyland & Sims, 1954). Both these amines have carbonyl groups *ortho* to the amino groups, and it is possible that the *meta*-directing influence of the carbonyl group has some effect on the position of substitution of the ester group. *o*-Aminoacetophenone also yielded two acid-labile products with persulphate, which are apparently the sulphuric esters of 2-amino-3- and 2-amino-5-hydroxyacetophenone, although their identity has not been conclusively established. These products were not detected among the products of the persulphate oxidation of tryptophan.

## SUMMARY

1. The actions of alkaline persulphate on DL-tryptophan, DL-kynurenine, 3-indolylacetic acid and indole have been examined, and its action on anthranilic acid has been re-examined. A number of products have been isolated or identified by means of paper chromatography.

2. All the above compounds yielded anthranilic acid, the sulphuric esters of 3-hydroxyanthranilic acid and *o*-aminophenol, and a product which is probably the sulphuric ester of 5-hydroxyanthranilic acid.

3. DL-Tryptophan also yielded formic acid and DL-kynurenine, together with products which are considered to be the sulphuric esters of 3- and 5-hydroxy-DL-kynurenine. These products were obtained in better yield from the oxidation of DL-kynurenine itself.

4. Indole gave indoxyl sulphate in good yield, identical with the naturally occurring ester.

We thank Professor A. Butenandt for the gift of synthetic 3- and 5-hydroxykynurenine, Dr C. E. Dalgliesh for the gift of some DL-kynurenine and Dr S. J. Holt for the gift of the *p*-toluidine salt of authentic indoxyl sulphate. We also thank Mr S. F. D. Orr for his help in carrying out the infra-red absorption spectra. Analyses are by Mr F. H. Oliver of the Organic Chemistry Department, Imperial College of Science and Technology. This work has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research, Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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# A Collimated Windowless Geiger Counter for Scanning Chromatograms

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(*Received* 13 *September* 1955)

The scanning of paper chromatograms and electrophoresis strips is a common requirement in biochemical laboratories using isotopes. A number of automatic apparatuses for doing this are available commercially in this country and use the standard E.H.M. (G.E.C. Research Laboratories) end-window counters. Collimation of the E.H.M.2.S. end-window counter to give a slit 5 mm. wide (which is desirable for analysing protein electrophoresis strips) reduces the sensitivity of such a counter by about 60%. A windowless counter with suitable geometry increases the sensitivity about three times. The counter described below was designed to combine the sensitivity of a windowless counter with narrow collimation and a low background.

Although a counter operating in the proportional region will tolerate contamination of counting gas with air, proportional counting necessitates additional expensive equipment which is not always available. A counter operating in the Geiger region (coupled to a standard rate meter) does not tolerate any contamination of counting gas with air, and an effective seal is therefore necessary.

## CONSTRUCTION

The dimensions and assembly of the counter are shown in Fig. 1. The counter body is made from drawn brass tubing 1·8 cm. in internal diameter, 2·5 cm. in external diameter and 8 cm. long, milled externally to give a flat surface 1·6 cm. wide; a window 3·0 cm. × 0·5 cm. is cut out in the middle of this flat surface. All surfaces and corners on the inside of the counter are polished smooth. The ends of the counter are of brass plate into which insulating polythene plugs are set. The anode (tungsten wire 0·002 in. in diameter) is shielded by brass sleeves, leaving an effective anode length of 2 cm. in the centre. A lead castle surrounds the upper surface and sides and projects 3 cm. beyond the ends of the counter. The castle is mounted on a brass plate which rests on collars surrounding the four locating studs screwed into the lead base.

An effective air-counting gas seal is achieved by the flat surface around the counter window resting on the paper strip, which is mounted on a Tufnol (Tufnol Ltd., Perry Barr, Birmingham, 22B) strip

resting on a sponge rubber pad covered with polythene. A thin wedge of rubber (not shown in Fig. 1) is inserted between the top of the counter and the castle. The pressure exerted by the counter on the paper can then be controlled by adjusting the two nuts threaded on the two brass rods passing vertically through the castle. The chromatogram strip should be wide enough to cover the whole of the counter becomes contaminated with dust. Should this happen the counter may be cleaned with compressed air.

The counter is coupled to a probe unit and a standard rate meter which records on a two-channel pen-recording ammeter. The speed of movement of the chromatogram and recording paper and the integrating time of the rate meter

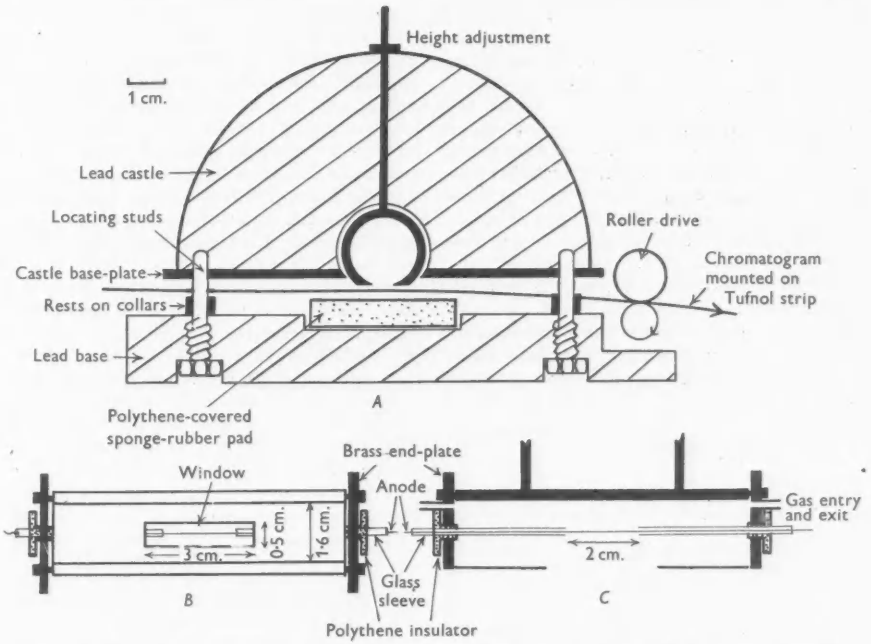


Fig. 1. *A*, Vertical cross-section of assembly; *B*, under-surface of counter; *C*, vertical section of counter.

width of the window. The Tufnol strip (10 cm. wide), on which the paper is mounted, is drawn between the collars and under the counter at 3·8 cm./hr. by a simple roller drive.

The counter operates with helium saturated with dry ethanol at 0° after 2–3 min. flushing. The flow rate thereafter should remain constant at about 1·0 l./hr. The counter has a plateau of 1300–1600 v with a slope of 4 %. With an operating potential of 1450 v the counter is stable from day to day, and scans of chromatograms are almost exactly reproducible. The background count is 6 counts/min., but it may rise slightly if the inside

depend on the degree of resolution required: a suitable combination for electrophoresis strips was found to be 3·8 cm./hr., 2·5 cm./hr. and 160 sec. (integrating capacity 8 $\mu$F) respectively.

## SUMMARY

A simple windowless helium–ethanol counter which operates in the Geiger region with a standard rate meter is described. It is designed to give narrow collimation and a low background suitable for scanning paper-chromatogram or -electrophoresis strips.

# A Simple Helium–Ethanol Flow Counter for Monitoring Chromatograph-Column Effluents Containing Weak β-Emitting Isotopes

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(*Received* 13 *September* 1955)

The conventional method for assay of weak β-emitting isotopes in the effluent from a chromatographic column is to dry down portions of fractions on planchettes, which are then counted under a standard thin-window E.H.M.2.S. (G.E.C. Research Laboratories) tube counter. This technique is laborious and slow, although sensitive and quantitatively relatively accurate. Other ways of monitoring the liquid as it comes off the column, involving the use of the E.H.M. tube, have been described, but they are relatively insensitive because of the small area (5 cm.²) of the end-window. In the counter described below sensitivity is increased by running the liquid as a thin film in contact with a thin window seven times the area of the E.H.M. window.

## CONSTRUCTION

The dimensions and assembly are apparent from Fig. 1. The window is of silicone-treated unsupported mica, which is replaceable, since the counter is easily dismounted. Mica sheets 25 cm. × 3 cm., 2·5 mg./cm.², are available from the Essex Mica Co., Hainault, Essex. The effective area of the window is $2{\cdot}5 \times 15 = 37{\cdot}5$ cm.².

The body of the counter in use is of stainless steel, but it may be made of brass, and the dimensions are not critical. The polythene gasket is thick enough to mount two small connecting tubes for the entry and exit of liquid. These holes are offset 1·5 cm. from the ends of the cell so that they are not immediately above unsupported mica; it is thought that this allows better liquid-flow characteristics. The polythene is very slightly recessed between the entry and exit tubes and the ends of the window, to allow liquid to flow through.

In assembly, after the counter has been cleaned carefully, a thin layer of silicone grease is smeared on the surface of the counter and the mica sheet laid on it over the cell. Another layer of grease is smeared over the edges of the mica, and the polythene gasket and backing plate are screwed on tightly. An effective seal (which is essential) is obtained by having milled flat steel surfaces, a rigid backing plate, a minimal amount of silicone grease and stout tightened screws. The liquid is thus in contact only with polythene and mica, and concentrated acids can be run through without corrosion. The liquid dead space is about 1·5 ml., including the entry and exit tubes; when a radioactive solution in the counter (giving 400 counts/min.) was washed through with 3 ml. of water a 95 % reduction in count rate was obtained, and after 10 ml. the count rate had fallen to background. N-HCl containing $^{14}$C-labelled piperazine compounds (Bangham, 1955) has been run through the counter over the course of 2 weeks, and no radioactive contamination was noticed.

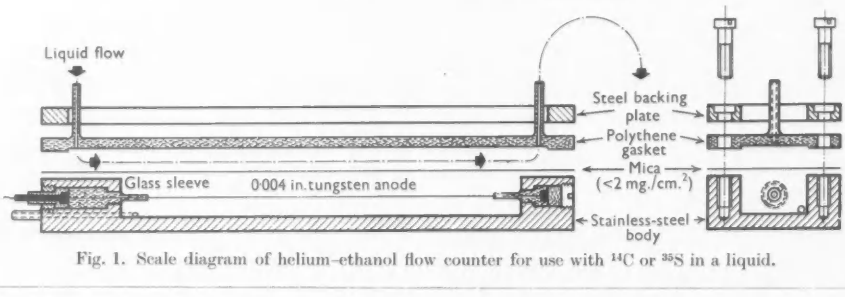


Fig. 1. Scale diagram of helium–ethanol flow counter for use with $^{14}$C or $^{35}$S in a liquid.

Helium bubbled through sodium sulphate-dried ethanol at 0° is passed through the counter cell at about 0·8 l./hr. This flow rate is not critical and

needs little attention. A lower rate is possible if a simple ethanol trap is connected to the exit pipe to prevent back-contamination with air.

The counter has a plateau of 1275–1775 v with a slope of 1·2 % per 100 v. With a working potential of 1400 v the counter is stable. The background is stable over long periods (about 50 counts/min. in a lead castle 1 in. thick).

The counter can be connected to a standard or log rate meter coupled to a pen recorder. A relay on the fraction-cutter siphon records each fraction on paper. The maximum rate of flow of the liquid depends on the radioactivity and the integrating time of the rate meter. With the geometry used and with $^{14}C$ in a liquid of specific gravity 1·0, approximately 1 % of disintegrations are recorded. This means that the counter gives the same number of counts as 0·2 ml. of the liquid evaporated and counted under an end-window Geiger–Müller tube. The counter must be calibrated each time it is reassembled, because the geometry of the liquid film may be altered. The pressure of the liquid should be kept constant.

The counter provides a continuous, immediately available record of radioactivity coming off a column, and it obviates a large number of tedious sampling functions. It is not intended as an instrument for accurate measurements.


## SUMMARY

A simple helium–ethanol flow counter suitable for indicating radioactivity due to weak $\beta$-emitting isotopes in the effluent from a chromatographic column is described.


I wish to thank Dr J. E. S. Bradley and Mr R. C. Holloway for their advice, Mr C. Dorè for constructing the counter, and Mr K. R. Hobbs for his technical assistance. This counter was exhibited at the Second International Isotope Conference at Oxford, 1954.

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# Studies in Carotenogenesis

## 20. CAROTENOIDS OF SOME SPECIES OF *CHLOROBIUM**

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(*Received* 10 *October* 1955)

No unequivocal information about the presence or absence of carotenoids in the green sulphur bacteria is available. van Niel & Muller (1931) failed to detect carotenoids in their cultures, but Katz & Wassink (1939) reported their presence in a different strain. It has now been possible to examine three species of *Chlorobium*; carotenoids were found in all and they have been separated and identified. A preliminary report of this work has already been given (Goodwin & Land, 1955). Barer (1955) has also recently mentioned the presence of $\gamma$-carotene in species of *Chlorobium*.

## EXPERIMENTAL

*Cultures.* The three species of *Chlorobium* examined were *C. thiosulphatophilum* (kindly supplied by Dr June Lascelles, Dept. of Biochemistry, The University of Oxford), *C. limicola*, and an unidentified halophilic species, both kindly supplied by Mr K. R. Butlin, Chemical Research Laboratory, D.S.I.R., Teddington, Middlesex.

*Culture medium.* The medium used was that recommended by Larsen (1952). For culturing the halophilic strain the NaCl concentration was increased from 1·0 to 2·5 % (w/v).

Diphenylamine media were made up from a quarter-saturated solution (1/140 000) of diphenylamine as the solvent instead of pure water.

*Cultural conditions.* The bacteria were grown anaerobically in completely filled glass-stoppered bottles at 30° in a glass incubator illuminated by two banks of four 60 w tungsten-filament lamps (Garton, Goodwin & Lijinsky, 1951). *Phycomyces blakesleeanus*, required as a source of $\gamma$-carotene, was grown under conditions described by Garton *et al.* (1951).

*Extraction and separation of pigments.* The centrifuged cells were first extracted twice with methanol to remove chlorophyll (Polgár, van Niel & Zechmeister, 1944; Goodwin & Osman, 1953) and then twice with ethyl ether. The combined methanol fractions were saponified by the method of Goodwin & Morton (1946) to remove chlorophyll and lipids that might interfere with chromatography. The unsaponifiable matter was extracted, combined with the original ethereal extract, dried by standing over anhydrous $Na_2SO_4$, evaporated to dryness *in vacuo* at 30° and redissolved in light petroleum (b.p. 40–60°) ready for chromatography (throughout this work the term light petroleum refers to a fraction with boiling point 40–60°).

The pigments were separated by chromatography on activated alumina (P. Spence and Co., Widnes, grade O),

* Part 19: Goodwin (1956).

grade O deactivated with methanol (Goodwin & Srisukh, 1949), an appropriate mixture of the two, or a MgO/Celite (2:1) mixture (Starr & Saperstein, 1953).

*Isomerization of pigments.* This was carried out by treatment with $I_2$, according to the method of Zechmeister (1944).

*Rubixanthin.* A chromatographically homogeneous specimen of this pigment was obtained from ripe rose hips (Kuhn & Grundmann, 1934) by the method of Goodwin (1956).

## RESULTS

### Chlorobium limicola

A typical chromatogram for the separation of the polyenes from *C. limicola* is given in Table 1. It will be seen that the pigment system consists virtually of two zones, 3 and 7. Zone 3 has been identified as $\gamma$-carotene by (*a*) its absorption spectrum with maxima at 435, 460 and 490·5 m$\mu$. in light petroleum and 450, 473 and 504·5 m$\mu$. in benzene and characteristic shape (Fig. 1); (*b*) the behaviour (hypophasic in the light petroleum/95 %, v/v, aqueous-methanol system) indicate that it is rubixanthin (monohydroxy-$\gamma$-carotene) (Kuhn & Grundmann, 1934). This has been confirmed by chromatographic comparison with a chromatographically homogeneous specimen of rubixanthin obtained from rose hips. When mixed, the two pigments could not be separated on columns of either weakened alumina or a mixture of MgO/Celite (2:1).

Zone 1, from its green fluorescence in ultraviolet light and absorption maxima [at 330 (inflexion), 348 and 368 m$\mu$.] contains traces of phytofluene. It occurs in such small quantities that it has not been possible to separate it completely from traces of a yellow material which run very closely with it on chromatography.

Zone 2 represents small amounts of pro-$\gamma$-carotene. Its absorption spectrum (Fig. 2) does not correspond exactly in shape with that of pro-$\gamma$-

Table 1. *Separation of polyenes of* Chlorobium limicola

Zones in order of increasing adsorptive affinity; adsorbent deactivated alumina; developer light petroleum containing varying amounts of ethyl ether.

| Zone no. | Description | Absorption maxima in light petroleum (m$\mu$.) | Concn. of ether (v/v) required for elution (%) | Relative amount of total polyene present (%) | Provisional identification |
|---|---|---|---|---|---|
| 1 | Very pale-yellow, slight green fluorescence | 348, 368, 430 | 0–1 | Trace | Mainly phytofluene |
| 2 | Pale orange | — | 1 | Trace | Pro-$\gamma$-carotene |
| 3 | Yellow-orange | 435, 460, 490·5 | 2–3 | 85 | $\gamma$-Carotene |
| 4 | Orange | 425, 448 (indefinite) | 6 | Trace | Indefinite |
| 5 | Yellow | 428 | 8 | Trace | Indefinite |
| 6 | Orange-pink | 446, 470, 500 | 10 | Trace | Indefinite |
| 7 | Orange | 435, 458, 487 | 12–15 | 12–15 | Rubixanthin |

inability to separate it chromatographically from an authentic specimen of $\gamma$-carotene from *Phycomyces blakesleeanus* (Goodwin, 1952*a*); (*c*) its very characteristic spreading on a column owing to ease of isomerization (Zechmeister & Schroeder, 1942); (*d*) its partition behaviour: it is epiphasic when partitioned between light petroleum and 95 % (v/v) aqueous methanol; (*e*) on isomerization by iodine changes characteristic of an all-*trans* carotenoid occur: the extinction, definition and position of the maxima all drop. The resulting absorption spectrum (Fig. 1) is very similar to that given by Zechmeister (1944) for isomerized $\gamma$-carotene, except that although the extinction of the isomerized pigment in the region 350–360 m$\mu$. is higher than that of the all-*trans* pigment, it has never been possible to obtain a marked *cis* peak at about 355 m$\mu$.

Zone 7 has the same absorption spectrum as $\gamma$-carotene, but its adsorptive affinity and partition

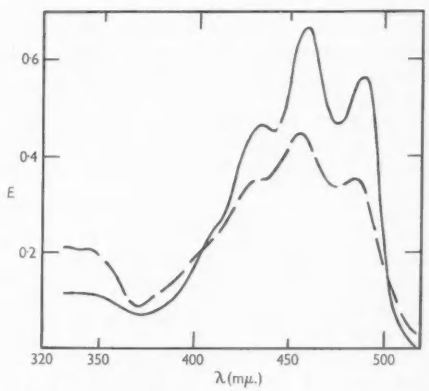


Fig. 1. Absorption spectrum of $\gamma$-carotene (fraction 3, Table 1) in light petroleum. Before isomerization, ——; after isomerization, — — —.

carotene described by Zechmeister & Schroeder (1942), mainly owing to contamination with traces of a pigment absorbing at slightly lower wavelengths. The minute traces available precluded complete purification. The reaction of this pigment with iodine is, however, very characteristic of pro-$\gamma$-carotene. Immediately on adding iodine there is a marked darkening in colour; the absorption spectrum increases in intensity, shifts very considerably to longer wavelengths and resembles closely the absorption spectrum of isomerized $\gamma$-carotene (Fig. 2). Isomerization of $\gamma$-carotene or

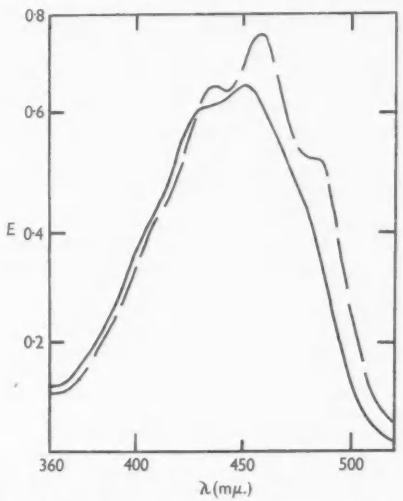


Fig. 2. Absorption spectrum of pro-$\gamma$-carotene (fraction 2, Table 1) in light petroleum. Before isomerization, ——; after isomerization, — — —.

lycopene with iodine does not yield any pro-$\gamma$-carotenes or prolycopene, but treatment of these procarotenes with iodine results in the production of the usual equilibrium mixture of these pigments (Zechmeister & Petracek, 1952). The extinction of the maximum at 435 m$\mu$. is slightly too high for isomerized $\gamma$-carotene (see Fig. 1), because of the presence of traces of the impurity just mentioned.

Zones 4–6 represent small traces of materials with indeterminate absorption spectra. They may be artifacts, and have not been examined in detail.

### Chlorobium thiosulphatophilum *and the halophilic* Chlorobium *species*

The pigments obtained from these other species examined were identical with those obtained from *C. limicola*, except that, in the halophilic strain, rubixanthin was either absent or present in much reduced quantities.

### *Action of diphenylamine*

Diphenylamine inhibits the synthesis of the normal carotenoid components in some members of the Thiorhodaceae and Athiorhodaceae (Goodwin & Osman, 1953, 1954; Goodwin & Land, unpublished observations); the synthesis of the more saturated polyenes—phytoene, phytofluene, $\zeta$-carotene and neurosporene—is, however, stimulated. At a level of 1/70 000 diphenylamine inhibits growth in all three species of *Chlorobium*. At a level of 1:140 000, however, it has qualitatively a similar effect to that just described. The synthesis of $\gamma$-carotene and rubixanthin is virtually completely suppressed. Phytoene and $\zeta$-carotene appear in diphenylamine cultures, but only in very small amounts; the amount of phytofluene is also slightly increased. This stimulation is quantitatively much less than that observed in the purple bacteria. Neurosporene has never been observed in diphenylamine cultures of *Chlorobium*, and this also applies to phytofluenol, hydroxy-$\zeta$-carotene and hydroxyneurosporene, which have recently been found in diphenylamine cultures of some purple bacteria (Goodwin & Land, unpublished observations).

Two unidentified colourless materials have also been observed in small amounts in diphenylamine cultures. One, non-fluorescent ($\lambda_{max.}$ 257 m$\mu$. in light petroleum), running very close to phytofluene, is probably the same as that observed in diphenylamine cultures of *Phycomyces blakesleeanus* (Goodwin, 1952*a*); the other, fluorescing blue ($\lambda_{max.}$ 274 m$\mu$. in light petroleum) is adsorbed between phytofluene and $\zeta$-carotene. These materials have not been investigated further.

## DISCUSSION

The occurrence of $\gamma$-carotene and its derivatives as the major carotenoids in *Chlorobium* is unique. These pigments do not occur in any other photosynthetic bacterium yet examined (Goodwin & Land, unpublished observations) and, with one exception, do not exist in the photosynthetic tissues of higher plants (Goodwin, 1952*b*). The exception is the leaf of the marsh dodder (*Cuscuta salina*), in which $\gamma$-carotene is the major carotenoid component (Mackinney, 1935). $\gamma$-Carotene was also thought to be absent from the Algae, but recently Strain (1951) has shown that it is present in trace amounts in a number of green algae (Chlorophyceae).

In general, however, $\gamma$-carotene can be considered to be a pigment much more characteristic of non-photosynthetic than of photosynthetic tissues, being widely distributed in fruit, flower petals and fungi, especially the last (Goodwin, 1952*b*). In non-

photosynthetic bacteria, however, its existence appears to be confined to the Mycobacteriaceae (Goodwin & Jamikorn, 1956).

The effect of diphenylamine on species of *Chlorobium* is qualitatively similar to its effect on *Rhodospirillum rubrum* (Goodwin & Osman, 1954) and a species of *Chromatium* (Goodwin & Land, unpublished observations), and different from its effect on *Rhodopseudomonas spheroides* (Goodwin, Land & Osman, 1955) and the algae *Chlorella vulgaris* (Goodwin, 1954) and *Euglena gracilis* v. *bacillaris* (Goodwin & Jamikorn, 1954), where growth and carotenoid synthesis are equally inhibited and there is no stimulation of synthesis of phytofluene etc.

## SUMMARY

1. *Chlorobium limicola*, *Chlorobium thiosulphatophilum* and an unnamed halophilic species produce $\gamma$-carotene as the main carotenoid pigment (85% of the total). Rubixanthin also occurs in the first two species (10–15% of the total). Traces of pro-$\gamma$-carotene and three unidentified pigments also exist; the last may be artifacts.

2. Diphenylamine strongly inhibits the synthesis of $\gamma$-carotene and its derivatives, but only slightly stimulates the production of phytoene, phytofluene and $\zeta$-carotene.

We thank the Medical Research Council for a grant toward laboratory expenses (T.W.G.) and the University of Liverpool for a fellowship (D.G.L.).

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# Studies on Cholinesterase

## 10. RETURN OF CHOLINESTERASE ACTIVITY IN THE RAT AFTER INHIBITION BY CARBAMOYL FLUORIDES*

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(*Received* 14 *September* 1955)

Eserine, prostigmine and analogous carbamates have, in contrast to the organophosphorus derivatives, generally been regarded as reversible inhibitors of the cholinesterases. However, a detailed study of the mechanism of inhibition of the pseudocholinesterase by a compound of this type (Myers, 1952) showed that the reaction was similar to that proposed for the inhibition by the organophosphorus derivatives (cf. Burgen, 1949; Burgen & Hobbiger, 1951; Wilson, 1951, 1952; Aldridge, 1950, 1953; Aldridge & Davison, 1952*a*, 1953). The inhibited enzyme is considered to be a dimethylcarbamoyl derivative for an inhibitor such as prostigmine (cf. Myers, 1954) and therefore analogous to the proposed dialkyl phosphoryl derivatives of the esterases. This conclusion was

* Part 9: Myers (1953).

confirmed by the finding that *NN*-dimethylcarbamoyl fluoride, like the dialkyl phosphonofluoridates, is also a potent inhibitor of the cholinesterases (Myers & Kemp, 1954).

On the basis of this theory, it would be expected that the return of the cholinesterase activity after inhibition *in vivo* should follow an exponential curve with the kinetics of a first-order reaction. This appears to be true for the pseudocholinesterase after inhibition by diethyl *p*-nitrophenyl phosphate (E 600) and analogues, but the activity of the pseudocholinesterase of brain returns much more slowly than that of other tissues (Davison, 1953, 1955). Moreover, the recovery of the true cholinesterase activity exhibits two distinct phases after inhibition by the organophosphorus derivatives; 60% of the activity returned much more rapidly than the remainder both *in vitro* and *in vivo*. Davison (1953, 1955) suggested that there are two types of true cholinesterase in the same tissue, differing in the stability of their dialkyl phosphoryl derivatives. Since the reaction of the carbamoyl fluorides and of the organophosphorus derivatives with the cholinesterases were presumed to follow the same pathways, it seemed interesting to investigate the return of cholinesterase activity in the rat after inhibition by carbamoyl fluorides. This investigation was carried out in the same way as has been described by Davison (1953, 1955) for the organophosphorus derivatives.

## METHODS

As in previous investigations, the esterase activity was measured manometrically by the Warburg technique at 37·5° and pH 7·4 in a medium containing 0·025 M-$NaHCO_3$ and saturated with $CO_2+N_2$ (5:95) (v/v). True cholinesterase activity was measured with 0·03 M acetyl-β-methylcholine (Mendel, Mundell & Rudney, 1943) and pseudocholinesterase activity with 0·02 M butyrylcholine as substrate (Cohen, Kalsbeek & Warringa, 1949). The aliesterases of serum and brain were determined with a 0·2% (v/v) emulsion of tributyrin stabilized with 0·1% gum acacia (final concentrations). Although the activities of these two ali-esterases are measured in the same way, it should be noted that the ali-esterase of serum is a different enzyme from that found in brain (Mendel & Myers, 1953; Mendel, Myers, Uyldert, Ruys & Bruyn, 1953; Myers & Kemp, 1954). The cholinesterases present in serum, on the other hand, appear to be identical with those found in other tissues (Ord & Thompson, 1952; Myers, 1953; Davison, 1953).

The inhibitors used were *NN*-dimethylcarbamoyl fluoride and *NN*-diethylcarbamoyl fluoride. Both compounds are colourless liquids, moderately soluble in water and stable in aqueous solution (Schrader, 1947). These compounds were injected in aqueous solution intraperitoneally into male rats weighing approx. 250 g. After various intervals of time the rats were killed under ether narcosis, the blood was collected from the carotid artery, the brains were removed and ground quickly with 4 vol. of cold water. The doses of these inhibitors were selected so as to produce fibrillary twitching of the muscles and other symptoms of acetylcholine accumulation due to true cholinesterase inhibition within a short time after the injection. Five of the forty-five rats injected with 5 mg./kg. of the dimethylcarbamoyl fluoride died 6–10 min. after the injection; the results for these animals were not included. In confirmation of the results obtained by Gunter & Mendel (1945), Koelle & Gilman (1946), Hawkins & Mendel (1947), Nachmansohn & Feld (1947) and Freedman & Himwich (1948), it was noted that the brains of the animals which succumbed to the inhibition contained less than 10% of their normal cholinesterase activity, whereas the brains of the surviving animals contained approx. 25% of the normal activity at this time.

Since the inhibition of the esterases by diethylcarbamoyl fluoride proved to be almost irreversible, it was not necessary to take any special precautions to minimize the reversal of the inhibition during the determination of the residual esterase activity. The inhibition by the dimethyl analogue, however, was more readily reversible. The brain suspension was, therefore, held at 2° until the esterase activity could be determined, this determination being carried out on the same day. Further, the volume of the reaction mixture in the Warburg vessels was restricted to 2 ml. to minimize the dilution of the brain, and the esterase activity was measured over a period of 20 min. after previous equilibration of the vessels for 15 min., in order to minimize the time of incubation at 37·5° *in vitro*. The experiments with serum were carried out in the same way but the serum was placed in the side arm of the Warburg vessel; this eliminated any dilution effects up to the time when the esterase activity was measured. This procedure was apparently fairly successful in minimizing the *in vitro* recovery of the inhibited esterases, since the cholinesterase of brain appeared to be 75–95% inhibited at a time when the symptoms of cholinesterase inhibition in the animal were at a maximum; these values are only slightly lower than those obtained with an irreversible inhibitor (cf. Nachmansohn & Feld, 1947; Freedman & Himwich, 1948). The small errors caused by a small reversal of the existing inhibition *in vitro* do not, in any case, have any influence on the estimation of the rate of recovery, since the time of incubation at 37·5° *in vitro* was identical in all experiments and thus the error introduced should be the same in all cases.

The rate of recovery of the esterase activity after inhibition *in vivo* can be estimated from the exponential curve obtained when the esterase activity is plotted against the time after injection of the inhibitor. More accurate data are obtained when the logarithm of the percentage inhibition is plotted against the time (cf. Aldridge, 1953; Davison, 1953, 1955). The half-life of the enzyme–inhibitor complex can be read from the straight line obtained and the first-order constant ($k_D$) for the decomposition of the enzyme–inhibitor complex calculated as $k_D = 0{\cdot}693/\text{half-life}$. In the present investigation it was found that the esterases, despite their difference in sensitivity to the inhibitors (Fig. 1), all began to recover exponentially at the same time, i.e. 10 min. after injection of the dimethylcarbamoyl fluoride and 3 hr. after injection of the diethyl analogue. The absence of a significant lag phase with one or two of the esterases and the large differences in the rate

of recovery of the various esterases indicate that the rate of recovery is not complicated by the presence of appreciable amounts of free inhibitor in the animal after these periods of time.

## RESULTS

Fig. 1 shows the relation between the inhibitor concentration and the percentage inhibition of the esterase activities after incubation for 30 min. at

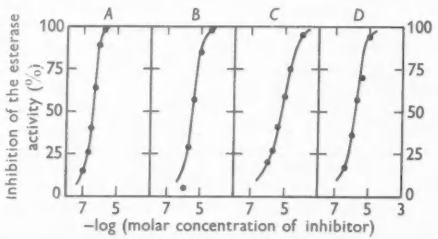


Fig. 1. Inhibition of the esterases of the rat by *NN*-dimethylcarbamoyl fluoride *in vitro*. The enzyme preparations were incubated for 30 min. at 37·5° and pH 7·4 with various concentrations of the inhibitor before measuring the residual esterase activity. Results are given for: *A*, the true cholinesterase of rat brain; *B*, the pseudocholinesterase of rat serum; *C* the ali-esterase of rat brain; *D*, the ali-esterase of rat serum.

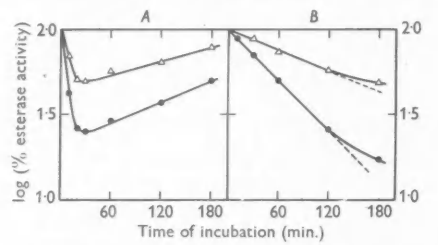


Fig. 2. Progress of the inhibitory reaction between the true cholinesterase of rat brain and the dialkylcarbamoyl fluorides *in vitro*. Rat-brain suspensions were incubated at 37·5° and pH 7·4 for various periods of time with a selected concentration of inhibitor before measuring the residual activity of the true cholinesterase. Data are given for: *A*, *NN*-dimethylcarbamoyl fluoride in concentrations of $4 \times 10^{-7}$M (△) and $1 \times 10^{-6}$M (●); *B*, *NN*-diethylcarbamoyl fluoride in concentrations of $2 \times 10^{-6}$M (△) and $1 \times 10^{-5}$M (●).

37·5° with dimethylcarbamoyl fluoride. The curves show no abnormal inflexions and are of the shape expected for a competitive esterase inhibitor (Goldstein, 1944). Similar curves were obtained with the diethylcarbamoyl fluoride.

Fig. 2 shows the progress of the inhibitory reaction between the true cholinesterase of rat brain and the two carbamoyl fluorides after various periods of incubation. The reaction with the dimethyl compound (Fig. 2*A*) reaches an equilibrium within 30 min., and subsequently some reversal of the inhibition is apparent as the incubation is prolonged. These results are strikingly similar to those reported by Aldridge (1953) for the inhibition of the true cholinesterase of whole-rabbit blood by dimethyl *p*-nitrophenyl phosphate; the reversal of the inhibition on prolonged incubation of the reaction mixture is probably due in both cases to destruction of the excess of inhibitor and subsequent recovery of the inhibited enzyme. On the other hand, the inhibition by diethylcarbamoyl fluoride increases continuously for several hours (Fig. 2*B*); similar results have been obtained for the inhibition of the true cholinesterase by diethyl *p*-nitrophenyl phosphate (Aldridge, 1950, 1953; Aldridge & Davison, 1952*a*).

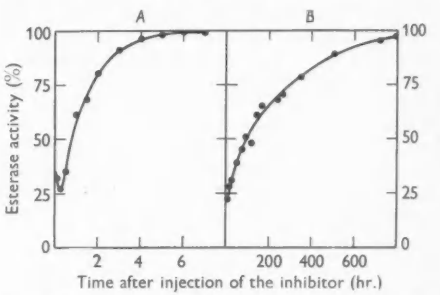


Fig. 3. Return of the activity of the true cholinesterase after inhibition by the dialkylcarbamoyl fluorides *in vivo*. The results given are those obtained after intraperitoneal injection of: *A*, 5 mg. of *NN*-dimethylcarbamoyl fluoride/kg.; *B*, 40 mg. of *NN*-diethylcarbamoyl fluoride/kg. Each point represents the average of individual measurements on two to six rats; further details are given in Table 1.

The return of the true cholinesterase activity in the brain of rats after injection of the carbamoyl fluorides is indicated in Fig. 3. The rates of recovery after inhibition by the dimethyl- and diethyl-carbamoyl fluorides are very similar to the rates of recovery of true cholinesterase activity after inhibition by dimethyl and diethyl *p*-nitrophenyl phosphates respectively (cf. Aldridge, 1953; Davison, 1953, 1955). However, the curve for the recovery of the true cholinesterase of rat brain after inhibition by the organophosphorus compounds shows a marked inflexion at the point of 60% activity, the remaining 40% of the activity being more permanently inhibited (Davison, 1953, 1955). The recovery of the same enzyme after inhibition by the carbamoyl fluorides, on the other

hand, continues to completion and appears to follow the exponential curve of a first-order reaction (Fig. 3). This supposition was confirmed by the fact that a straight line is obtained when the logarithm of the percentage inhibition is plotted against the time (Fig. 4). From this line, the half-life periods of the inhibited cholinesterase were estimated to be 0·88 and 182 hr. after inhibition by the dimethyl- and diethyl-carbamoyl fluorides




Fig. 4. Return of the activity of the true cholinesterase of rat brain plotted as a first-order reaction. The experimental results are the same as those of Fig. 3 and were obtained after injection of: *A*, 5 mg. of *NN*-dimethylcarbamoyl fluoride/kg.; *B*, 40 mg. of *NN*-diethylcarbamoyl fluoride/kg.

respectively. The corresponding first-order constants ($k_D$) for the decomposition of the enzyme–inhibitor complexes were calculated as $1{\cdot}3 \times 10^{-2}$ and $6{\cdot}3 \times 10^{-5}$ min.$^{-1}$ respectively (Table 1). By way of comparison, it might be noted that the first-order constant for the recovery of the true cholinesterase of rabbit erythrocytes after inhibition by dimethyl phosphoryl derivatives is about $8{\cdot}6 \times 10^{-3}$ min.$^{-1}$ (Aldridge, 1953).

The results obtained with the other esterase activities of brain and serum after injection of the carbamoyl fluorides are summarized in Table 1. These results indicate that a structural alteration which increases the stability of the inhibitor also tends to increase the stability of the enzyme–inhibitor complex. The inhibition of the true cholinesterase, pseudocholinesterase and serum aliesterase by the diethylcarbamoyl fluoride lasts 200, 70 and 10 times respectively longer than the inhibition of the same enzymes by the dimethyl analogue. This factor is usually apparent also in the results obtained with the organophosphorus esterase inhibitors (cf. Hobbiger, 1951; Burgen & Hobbiger, 1951; Wilson, 1952; Aldridge, 1953; Davison, 1955).

Table 1. *Recovery of the esterases of the rat after inhibition by dialkylcarbamoyl fluorides* in vivo

Male rats were injected intraperitoneally with 5 mg. of *NN*-dimethylcarbamoyl fluoride/kg. and with 40 mg. of *NN*-diethylcarbamoyl fluoride/kg. Groups of two to six rats were killed after various periods of time ranging from 6 min. to 40 days and the esterase activities measured as described in the text. The average results were plotted graphically as shown in Figs. 3 and 4 in order to determine the half-life of the inhibited enzyme. The first-order constant for recovery of the inhibited enzyme = 0·693/half-life.

| | | | | Inhibition by dimethylcarbamoyl fluoride | | | Inhibition by diethylcarbamoyl fluoride | | | First-order constant ($k_D$) for recovery of the inhibited enzyme (min.$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Enzyme | Source | No. of normal animals | Normal activity (μl. $CO_2$/g./30 min.) | Maximum inhibition (%) | No. of animals injected | Half-life of the inhibited enzyme (hr.) | Maximum inhibition (%) | No. of animals injected | Half-life of the inhibited enzyme (hr.) | Dimethylcarbamoyl derivative | Diethylcarbamoyl derivative |
| True cholinesterase | Brain | 44 | 1835 | 73 | 39 | 0·88 | 78 | 67 | 182 | 0·013 | 0·000063 |
| True cholinesterase | Serum | 10 | 64 | 99 | 37 | 0·90 | — | — | — | 0·013 | — |
| Pseudocholinesterase | Brain | 9 | 380 | 77 | 29 | 1·06 | — | — | — | 0·011 | — |
| Pseudocholinesterase | Serum | 31 | 238 | 88 | 37 | 1·12 | 90 | 52 | 80 | 0·010 | 0·00014 |
| Ali-esterase I | Brain | 32 | 2240 | 10 | 10 | — | 65 | 61 | 77 | — | 0·00015 |
| Ali-esterase II | Serum | 28 | 2410 | 95 | 37 | 5·80 | 98 | 52 | 63 | 0·0020 | 0·00018 |

The figures given in Table 1 also show that the true cholinesterase activities of brain and serum recover at approximately the same rate after inhibition by dimethylcarbamoyl fluoride. The same is true for the pseudocholinesterase activities of brain and serum, whereas the pseudocholinesterase activity of the brain recovers much more slowly than that of serum and other tissues after inhibition by the organophosphorus derivatives (Davison, 1953, 1955).

## DISCUSSION

The effect of the carbamoyl fluorides on the cholinesterases resembles that of the organophosphorus derivatives in many respects, as noted above. It seems probable that the enzyme–inhibitor complex can be represented as a dialkylcarbamoyl derivative of the enzyme active centre and that the mechanism of the inhibitory reaction is therefore similar to that proposed for the organophosphorus esterase inhibitors. This conclusion is substantiated by the results of a previous investigation on the prostigmine analogue Ro2–0683 (Myers, 1952), by the structure of the inhibitors used in the present investigation, by the prolonged esterase inhibition observed after injection of diethylcarbamoyl fluoride, and by the fact that the esterase activities recover exponentially with a first-order rate constant that is characteristic of the esterase in question and of the structure of the inhibitor.

The complete reaction between the esterase and inhibitor probably involves several stages, which could be written as follows (cf. Burgen & Hobbiger, 1951; Wilson, 1951; Aldridge, 1953):

$$EH + I_{ab} \underset{2}{\overset{1}{\rightleftharpoons}} EH\text{–}I_{ab} \rightarrow EI_a + HI_b$$

and $$EI_a + H_2O \underset{}{\overset{3}{\rightleftharpoons}} EI_a\text{–}H_2O \rightarrow EH + I_aOH,$$

where EH is the active esterase, $I_{ab}$ is the intact inhibitor molecule, $I_a$ the dialkylcarbamoyl or dialkyl phosphoryl moiety of the inhibitor and $I_b$ the remainder of the inhibitor, in this case fluoride. The Michaelis constant $K_I = (k_2 + k_D)/k_1$, where $k_1$ is the rate constant for the formation of enzyme–inhibitor complex, $k_2$ the rate constant for the reversal of this reaction, and $k_D$ the rate constant for the destruction of the inhibitor by the enzyme (Briggs & Haldane, 1925; cf. Michaelis & Menten, 1913). Since the compound $EI_a$ appears to be the stable form of enzyme–inhibitor complex, the values of $k_1$ and $k_D$ might well represent the rate constants for the particular reactions which limit the rates of formation and decomposition respectively of $EI_a$. However, the intermediary compounds $EH\text{–}I_{ab}$, $EI_a$ and $EI_a\text{–}H_2O$ all represent enzyme which is combined with inhibitor and the experimental methods only distinguish inhibited enzyme from the enzyme which is free to react with the substrate. The rate constants for the intermediary reactions involving the conversion of one form of inhibited enzyme into another may therefore never appear in the measured value of $K_I$; in this case, the measured value of $k_1$ will represent the rate constant for reaction 1 in the scheme given above, $k_2$ that for reaction 2 and $k_D$ the rate constant for reaction 3.

The values of $k_D$ and $k_1$ have been measured for the reactions between the cholinesterases and a number of organophosphorus derivatives (Aldridge & Davison, 1952*a*, 1953; Aldridge, 1953; Davison, 1953, 1955). The comparable values of $k_D$ for the four esterases and the two carbamoyl fluorides tested in the present investigation are given in Table 1. The initial inhibitory reaction between the esterases and the carbamoyl fluorides is complicated by the instability of the inhibitors in the reaction mixtures containing brain or serum; however, the initial reaction between the true cholinesterase of rat brain and diethyl carbamoyl fluoride (Fig. 2*B*) does seem to be pseudo-monomolecular, as would be expected for a reaction of the form

$$E + I \rightarrow EI,$$

where the inhibitor is present in large excess. This would indicate that the value of $k_2 + k_D$ must be small; $k_1$ can therefore be determined from the equation $k_1 = -\ln a/It$, where $a$ represents the fractional enzyme activity, $I$ the molar concentration of the inhibitor and $t$ the time of incubation (cf. Aldridge, 1950; Aldridge & Davison, 1952*b*). In the case given above $k_1 = 1{\cdot}2 \times 10^3$ l. mol.$^{-1}$ min.$^{-1}$, and therefore

$$K_I = (k_2 + k_D)\, k_1 \geqslant k_D/k_1 = 5 \times 10^{-8} \text{ mol. l.}^{-1}.$$

Similar calculations for the initial rate of the reaction between the true cholinesterase of rat brain and dimethylcarbamoyl fluoride (Fig. 2*A*) indicate that $k_1$ must have a value of

$$1{\cdot}0\text{–}1{\cdot}5 \times 10^5 \text{ l. mol.}^{-1} \text{ min.}^{-1}$$

and therefore that $K_I \geqslant 10^{-7}$ mol. l.$^{-1}$.

These values for $K_I$ can be regarded only as minimum estimates, since there is no experimental proof for the assumption that $k_2$ is very small in comparison with $k_D$ in the carbamoyl fluorides. An analysis of the rate constants for the reaction between the pseudocholinesterase of human serum and the prostigmine analogue Ro2–0683 showed that the value of $K_I$ was equal to $k_D/k_1$ in this case. However, according to the present concept, the active centres of both cholinesterases possess an anionic site which increases the affinity of these

enzymes for choline esters and structurally related inhibitors (cf. Myers, 1951; Bergmann & Segal, 1954); thus it is conceivable that $k_2$ might have a negligible value only when the inhibitor or substrate contains a cationic ammonium group in a suitable position for combining with the anionic site of the active centre of the cholinesterases. This is true for prostigmine and its analogue Ro2–0683, but not for the carbamoyl fluorides. A fact that does not support this suggestion is that it is possible to obtain 50% inhibition of the true cholinesterase of rat brain with $4 \times 10^{-7}$ M dimethylcarbamoyl fluoride Fig. 1 (Myers & Kemp, 1954); since this represents the maximum possible value of $K_I$ and the minimum value calculated above is about $1 \times 10^{-7}$ M, it is evident that $k_2$ could not in any case be much greater than $k_p$. Similarly, Aldridge (1950) and Aldridge & Davison (1952*b*) were not able to obtain evidence for reversal of the inhibition by reaction 2 in experiments with organophosphorus derivatives which did not contain a cationic ammonium group.

The only marked dissimilarity between the carbamoyl fluorides and the organophosphorus derivatives in their effects on the cholinesterase appears in the rate of recovery of the cholinesterases of the brain after inhibition by these compounds. The difference between the rates of recovery of the pseudocholinesterase activities of brain and serum and the difference in the rates of recovery of the two fractions of the true cholinesterase activity in the brain observed after inhibition by the organophosphorus derivatives (Davison, 1953, 1955) fail to appear after inhibition by the carbamoyl fluorides.

If there are two forms of the true cholinesterase in the same tissue, and two forms of the pseudocholinesterase in different tissues of the same animal, as suggested by Davison (1953, 1955), it is evident that the differences between the two forms must be very small. Apparently both forms of the same enzyme react at the same rate with carbamoyl or with organophosphorus derivatives; there are no marked differences in the sensitivity to the inhibitors (Ord & Thompson, 1950, 1952; Davison, 1953) and no abnormal inflexions are observed in the curves obtained when the percentage inhibition of the total true cholinesterase activity of brain is plotted against the concentration of the inhibitor. Further, the relative rate of hydrolysis of various choline esters appears to be identical, even when the rate of recovery after inhibition by organophosphorus derivatives is different (Ord & Thompson, 1952; Davison, 1953). These results, together with the observations made in the present investigation, all suggest that the structure of the enzyme active centre is identical for the two hypothetical forms of the enzyme in question. It seems probable, therefore, that any difference between the two forms of the true cholinesterase or of the pseudocholinesterase must be ascribed to groups on the enzyme surface adjacent to the enzyme active centre rather than to the enzyme active centres themselves.

In this connexion it might be noted that the hydrolytic activity of the esterases seems to be due to an imidazole group, probably a histidine residue (Wilson & Bergmann, 1950; Wilson, 1952; Wagner-Jauregg & Hackley, 1953; Weil, James & Buchert, 1953; Weil & Seibles, 1955). The organophosphorus derivatives, the carbamoyl derivatives and the carboxylic acid esters, which serve as substrates, all presumably react with this imidazole group in the same way. It is assumed at present that the acylhistidine derivatives, which are formed during the reaction with carboxylic acid esters (e.g. acetylcholine), undergo a spontaneous hydrolysis without further intramolecular rearrangement. In this case it might logically be assumed that the carbamoylhistidine derivatives behave similarly to the acyl derivatives, since they have a similar structure. However, the dialkyl phosphoryl derivatives of the enzyme active centre may subsequently undergo a partial intramolecular rearrangement whereby the dialkyl phosphoryl group is transferred to a serine residue (Wagner-Jauregg & Hackley, 1953; Schaffer, May & Summerson, 1953, 1954; Oosterbaan, Kunst & Cohen, 1955). We suspect that the difference in the rate of recovery of the two fractions of the true cholinesterase activity after inhibition by the organophosphorus derivatives is due to the existence of these two forms of dialkyl phosphorylated enzyme.

Since the dialkyl phosphoryl serine derivative appears to be considerably more stable than the histidine derivative which is formed initially (cf. Schaffer *et al.* 1953, 1954; Cunningham, 1954; Jandorf, Crowell & Levin, 1955; Hobbiger, 1955), it seems likely that the more permanently inhibited portion of the true cholinesterase activity probably represents a dialkyl phosphoryl serine derivative. The readily reversible portion of the activity might then represent the dialkyl phosphoryl histidine derivative. In this case, it would be necessary to assume that the transfer of the dialkyl phosphoryl group to the serine residue is prevented by shielding or absence of the serine residue adjacent to the enzyme active centre.

A different explanation for the disparity in the rates of recovery of the two fractions of true cholinesterase activity in rat-brain preparations after inhibition by organophosphorus derivatives is suggested by the effects of proteolytic enzymes observed by Davison (1955). The slowly reversible portion of the true cholinesterase activity of brain was much more resistant than the rapidly reversible

portion to the action of pepsin. Moreover, the rate of recovery of pseudocholinesterase after inhibition by organophosphorus derivatives was increased in the presence of trypsin. It is possible, therefore, that the rates of recovery of the true cholinesterase and pseudocholinesterase activities *in vivo* and in crude tissue preparations *in vitro* depend partly on the action of the intracellular proteolytic enzymes, namely cathepsins. It might then be assumed that the cholinesterase activities of brain which recover slowly after inhibition by the organophosphorus derivatives represent lipoprotein complexes which are not accessible to the action of these proteolytic enzymes and which recover at the normal rate for the dialkyl phosphoryl serine derivatives.

The experimental data available at present do not allow a decision on the validity of the two explanations suggested. However, in view of the considerations given above, the conclusion that the slowly reversible and the rapidly reversible fractions of the cholinesterase activities are due to different enzymes does not seem to be justified. Both fractions appear to possess identical active centres and recover their activity at the same rate after inhibition by the carbamoyl fluorides; where differences in the rate of recovery of the two fractions are observed, they could be ascribed, directly or indirectly, to migration of the inhibitory dialkyl phosphoryl group from the enzyme active centre to an adjacent serine residue.

## SUMMARY

1. The initial inhibitory reaction between the true cholinesterase of rat brain and *NN*-dimethyl- or *NN*-diethyl-carbamoyl fluoride is similar to that observed with the organophosphorus esterase inhibitors. The reaction with the dimethyl compound reaches an equilibrium quickly, whereas the inhibition by the diethyl compound increases continuously for several hours.

2. After injection of the carbamoyl fluorides into rats, the esterase activities recover completely; the recovery follows the exponential curve of a first-order reaction. The rate of recovery is independent of the tissue in which the esterase is located but differs from one esterase to another. All four esterases investigated recovered more slowly after inhibition by the diethyl compound than after inhibition by the dimethyl compound.

3. An attempt was made to analyse the reaction of the inhibitors with cholinesterase in terms of the rate constants involved in the Michaelis constant $K_1=(k_2+k_D)/k_1$ (cf. Briggs & Haldane, 1925). It is suggested that $k_2$ may not have a large value.

4. The results support the conclusion that the mechanism by which the carbamoyl derivatives inhibit the esterases is similar to that proposed for the organophosphorus derivatives. The differences in the return of the cholinesterase activity after inhibition by the carbamoyl and by the organophosphorus derivatives are discussed in terms of the structure of the enzyme active centre.

The author is indebted to Miss E. G. L. Simons for technical assistance and to Dr G. Schrader, Elberfeld, for samples of *NN*-dimethylcarbamoyl fluoride and *NN*-diethylcarbamoyl fluoride.

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# The Formation of Glyoxylate and Succinate from Tricarboxylic Acids by *Pseudomonas aeruginosa*

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(*Received* 22 *July* 1955)

Campbell, Smith & Eagles (1953), in a brief note, reported that cell-free extracts of *Pseudomonas aeruginosa* convert citrate and *cis*-aconitate, but not *iso*citrate, into succinate and glyoxylate. The glyoxylate was identified chromatographically as the 2:4-dinitrophenylhydrazone. They also claimed that the reaction was reversible, since incubation of the extract with a mixture of succinate and glyoxylate gave rise to citrate.

The work described below, a preliminary report of which has already been published (Saz, 1954), confirms and extends these findings. Recently, Olson (1954) has shown that *Penicillium chrysogenum* contains a similar enzyme system.

## MATERIALS AND METHODS

*Organisms.* *Pseudomonas fluorescens* strain KB1 (Kogut & Podoski, 1953) and a laboratory strain of *Ps. aeruginosa* were used. Stock cultures were maintained on slopes containing 2% of Davis New Zealand agar and 10% (v/v) of yeast autolysate (Barker & Beck, 1942). For use the organisms were cultured as follows: Pyrex penicillin culture flasks containing 300 ml. of 10% (v/v) yeast autolysate were inoculated with 1 ml. of a suspension prepared by emulsifying the overnight growth on a slope in 4 ml. of sterile distilled water. The flasks were incubated for 20 hr. at 25° on a reciprocating shaking machine. The cells were washed thrice in distilled water before use. Stock cultures were maintained on agar slopes.

*Reagents.* Sodium glyoxylate was synthesized as described by Weissbach & Sprinson (1953), and the product assayed for glyoxylic acid by the bisulphite-binding method of Long (1942) with the correction factor recommended by this author. (±)-*iso*Citrate was prepared by alkaline hydrolysis of trichloromethylparaconic acid. (+)-*iso*Citric acid was a sample isolated from blackberry leaves and generously supplied by Professor H. A. Krebs. An aqueous extract of boiled brewer's yeast was prepared by suspending 1 g. of dried brewer's yeast (prepared from yeast obtained from Tennant Bros. Ltd., Sheffield) in 10 ml. of distilled water and heating for 10 min. in a boiling-water bath with occasional stirring. The suspension was then cooled in an ice bath and clarified by centrifuging. The clear brownish supernatant so obtained was stored at −20°. Ashed boiled brewer's yeast was prepared by evaporating a sample in a porcelain crucible; when the sample was dry the crucible was closed with a loosely fitting lid and heated for 1 hr. over a Bunsen burner. The cooled residue was suspended in a volume of distilled water equal to the volume of the sample of boiled brewer's yeast taken. All other reagents were of A.R. quality and obtained commercially.

*Analytical methods.* Paper-partition chromatography of 2:4-dinitrophenylhydrazones was carried out by the method of Cavallini, Frontali & Toschi (1949) with Whatman no. 1 filter paper. The papers were developed by the descending method, with *n*-butanol saturated with water as the solvent. Glyoxylate was estimated by the method for 'total keto acids' devised by Friedemann & Haugen (1943). This was considered justifiable since, as will be shown below, paper-partition chromatography indicated that glyoxylate was the only keto acid produced by the enzyme system. Succinate was estimated either by partition chromatography on ether-washed Celite 545 (Johns-Manville Co. Ltd., Artillery Row, London, S.W. 1) as described by Swim & Krampitz (1954) or by the enzymic method of Krebs (1937). Citrate was estimated either colorimetrically by the method of Taylor (1953) or chromatographically as described by Swim & Krampitz (1954).

### *Enzyme preparations*

'*Mickle*' *extracts.* The washed cells were suspended in cold (5°) distilled water (4 ml./g. of cell paste) and the suspension shaken in a Mickle disintegrator (Mickle, 1948)

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with Ballotini beads, grade 12, for 30 min. at room temperature. The glass beads were filtered off through a sintered-glass funnel, porosity 2, and the filtrate was centrifuged for 15 min. at 11 500 rev./min. in an M.S.E. High-Speed Angle Centrifuge '13' (Measuring and Scientific Equipment Ltd., Spenser Street, London, S.W. 1) at room temperature. The supernatant was stored at −20°.

*Hughes-press extracts.* The paste of washed cells was intimately mixed with powdered Pyrex glass (0·5 g./g. of cell paste), the mixture was transferred to a cooled Hughes press and the cells were frozen and crushed as described by Hughes (1951). The crushed cells were removed from the press, mixed with 3 ml. of cold (0°) distilled water/g. of cells and centrifuged for 15 min. at 11 500 rev./min. in the high-speed centrifuge. The supernatant was stored at −20°.

*Whole dried cells.* The washed cell paste was spread in thin layers on glass Petri dishes. The cells were then desiccated *in vacuo* over concentrated $H_2SO_4$ for 49 hr. When required the dried cells were suspended in water (30 mg./ml.) and 0·5 ml. of the suspension was added to each Warburg vessel.

*Fractionation with ammonium sulphate.* The extracts, prepared as described above, were cooled in an ice bath and solid ammonium sulphate was added slowly to the desired concentration with continuous stirring. The precipitate which formed at 30% saturation was centrifuged down and discarded. The precipitate which formed at 30–60% saturation was centrifuged down and dissolved in cold distilled water; (0·5 ml. of water/ml. of original crude extract) and stored at −20°. Most of the activity was contained in this fraction and the supernatant was discarded.

All incubations were carried out in Warburg respirometers. Usually aminotrishydroxymethylmethane (tris) was employed as buffer. N-$H_2SO_4$ was added to M tris until pH 8·0 was reached. Unless otherwise stated, 0·05 ml. of this buffer solution was added/ml. of reaction mixture in the Warburg vessel.

## RESULTS

*Products of the reaction.* When citrate, *cis*-aconitate or (±)-*iso*citrate was incubated with cell-free preparations of *Ps. fluorescens* KB 1 or *Ps. aeruginosa*, a compound was produced which formed a 2:4-dinitrophenylhydrazone. Paper chromatography of this derivative by the method of Cavallini *et al.* (1949) gave two spots. The derivative prepared from synthetic glyoxylic acid also gave two spots with the same $R_F$ values, and no separation was achieved on chromatographing a mixture of the synthetic and natural products. Stewart (1953) has also observed that two spots are formed on chromatographing glyoxylic acid 2:4-dinitrophenylhydrazone; presumably these are due to the two geometrical isomers of this compound. The same 2:4-dinitrophenylhydrazone was produced from all three tricarboxylic acids by extracts of the two species of *Pseudomonas* used. The 2:4-dinitrophenylhydrazone was isolated in crystalline form as follows. To 3 ml. of a Hughes-press extract were added 1 ml. of tris buffer, pH 8·0, 500 μmoles of *cis*-aconitate and water to 9 ml. in a large Warburg vessel (capacity 125 ml.). The vessel was gassed with $N_2$ and incubated at 30° with constant shaking. After 90 min. the contents were deproteinized by the addition of 3 ml. of 10N-$H_2SO_4$. The precipitated protein was centrifuged and washed once with a small volume of water. The combined supernatants were treated with 6 ml. of a 2%(w/v) solution of 2:4-dinitrophenylhydrazine in 6N-$H_2SO_4$. The precipitate which formed after standing overnight at 5° was filtered off and redissolved in a small amount of ether. The ethereal solution was extracted with an equal volume of 10% (w/v) $Na_2CO_3$. The aqueous layer was removed and acidified with 3N-HCl. An immediate precipitate formed which was filtered off, redissolved in ether and re-extracted with 10% $Na_2CO_3$. After separation and acidification of the aqueous layer, the 2:4-dinitrophenylhydrazone was filtered off, washed with cold water and dried *in vacuo* over calcium chloride. The derivatives of the biologically formed keto acid and synthetic glyoxylic acid both decomposed at 188–189°. Brady (1931) reported a decomposition point of 190° for this derivative of glyoxylic acid.

Table 1 shows some of the results obtained with various preparations of *Ps. aeruginosa*. Qualitative studies with *Ps. fluorescens* KB 1 demonstrated that Mickle extracts of this organism also formed glyoxylic and succinic acids from the tricarboxylic acids. However, these preparations had approximately only one-third of the activity of similar extracts prepared from *Ps. aeruginosa*.

Table 1. *Glyoxylate production from tricarboxylic acids by preparations of* Pseudomonas aeruginosa

Each Warburg flask contained 0·5 ml. of enzyme preparation, 0·1 ml. M tris buffer (pH 8·0) and 30 μmoles of substrate; total vol., 2·0 ml.; incubated for 60 min. at 30° in $N_2$. Amounts of glyoxylate formed are expressed in μmoles.

| | Glyoxylate formed from | | |
|---|---|---|---|
| Enzyme preparation | Citrate | (+)-*iso*-Citrate | *cis*-Aconitate |
| Suspension of dried cells | 6·61 | 6·41 | 8·04 |
| Mickle extract | 5·0 | — | 6·61 |
| Hughes-press extract | 6·9 | 8·26 | 7·82 |

The evidence that the other product of the reaction is succinate is as follows. It was oxidized by the minced heart-muscle preparation of Krebs (1937), which is reasonably specific for succinate, and in addition it behaved identically with succinate on the Celite column of Swim & Krampitz (1954).

Table 2 shows the stoicheiometry of the reaction. In this experiment *cis*-aconitate was the substrate.

It will be seen that glyoxylate and succinate were formed in approximately equivalent amounts and that a large amount of citrate was also produced, which indicates the presence of a powerful aconitase in the Hughes-press extracts of *Ps. aeruginosa*. In a similar experiment with citrate as substrate and the Mickle extract of *Ps. aeruginosa*, 3·2 μmoles of citrate disappeared while 3·1 μmoles of succinate and 3·0 μmoles of glyoxylate accumulated. These results are in agreement with those recorded in Table 2.

Table 2. *Stoicheiometry of the enzymic breakdown of* cis-*aconitate by extracts of* Pseudomonas aeruginosa

Each Warburg flask contained 0·3 ml. of Hughes-press extract of *Ps. aeruginosa*, 0·1 ml. of M tris buffer (pH 8·0) and 24·6 μmoles of *cis*-aconitate (Na salt); total vol., 2·0 ml. Incubation for 90 min. at 30° in $N_2$.

| | Amount formed (μmoles) | Amount formed (μatoms of carbon) |
|---|---|---|
| Citrate | 15·9 | 95·4 |
| Succinate | 6·0 | 24·0 |
| Glyoxylate | 6·8 | 13·6 |
| Total carbon recovered | — | 133 |
| Initial *cis*-aconitate | 24·6 | 147·6 |

Carbon recovery = 90%.

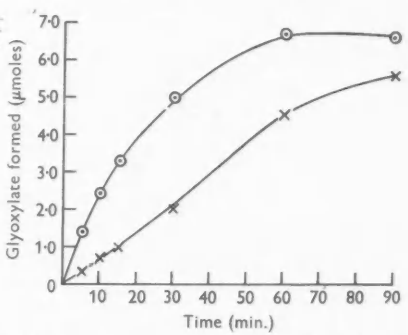



Fig. 1. Time course of glyoxylate formation from *cis*-aconitate. Reactions carried out in Warburg manometers in $N_2$. Each vessel contained 0·1 ml. of M tris buffer (pH 8·0), 30 μmoles of *cis*-aconitate, stated amount of enzyme preparation (Hughes-press extract) of *Ps. aeruginosa* and water to 2·0 ml.; temp. 30°. ⊙, 0·3 ml. of enzyme; ×, 0·1 ml. of enzyme.

*Kinetics.* Fig. 1 shows progress curves for glyoxylate formation from *cis*-aconitate with two different enzyme concentrations. In this experiment two series, each of six Warburg flasks, were set up, one containing 0·3 ml. of a Hughes-press extract of *Ps. aeruginosa*, the other 0·1 ml. of the extract; in all other respects the composition of the flask contents was identical. At suitable intervals a manometer was removed from the bath and the reaction stopped by the addition of 0·3 ml. of 10 N-$H_2SO_4$ and samples were taken for the estimation of glyoxylate. It will be seen that the rate of the reaction was linear for only a short period and thereafter fell off rapidly. Regardless of the enzyme concentration only a small amount of substrate was converted into glyoxylate; in the experiments shown in Fig. 3 the amount of glyoxylate formed at the end of the experiment was equivalent to less than 25% of the *cis*-aconitate added (30 μmoles).

In experiments of short duration (10 min.) the rate of glyoxylate formation was proportional to the enzyme concentration (Fig. 2); *cis*-aconitate was again the substrate and the enzyme preparation was a Hughes-press extract of *Ps. aeruginosa*.

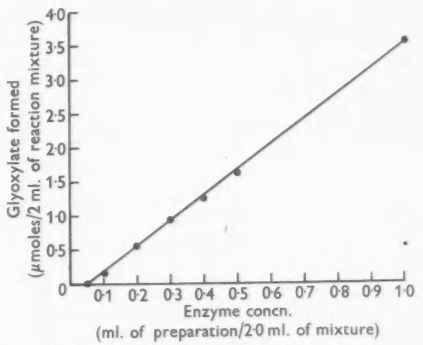



Fig. 2. Effect of enzyme concentration on glyoxylate formation from *cis*-aconitate. Reactions carried out in Warburg manometers in $N_2$. Each vessel contained 0·1 ml. of M tris buffer (pH 8·0), 30 μmoles of *cis*-aconitate, stated amount of enzyme and water to 2·0 ml.; temp. 30°; duration of experiment 10 min.

Although the optimum pH for the reaction varied somewhat with the conditions of assay and the buffer employed, the maximum activity was generally obtained at a pH of approximately 8.

*Reversibility of the reaction.* Extracts of *Ps. aeruginosa*, prepared with the Mickle disintegrator, failed to synthesize citrate on incubation anaerobically with a mixture of succinate and glyoxylate, but when the experiments were repeated with extracts prepared with the Hughes press, citrate was formed. The results of a typical experiment are given in Table 3. No citrate was formed in the absence of substrate; indeed the presence of both succinate and glyoxylate was essential for the production of the tricarboxylic acid, and even then only a small fraction reacted. Since the enzyme

preparation contained a powerful aconitase this experiment does not necessarily mean that citrate was the first product.

*Cofactor requirements.* The formation of glyoxylate from citrate, *iso*citrate and *cis*-aconitate was inhibited by ethylenediaminetetraacetic acid (EDTA) and pyrophosphate and by high concentrations of cyanide. Fig. 3 shows the effect of varying concentrations of EDTA and pyrophosphate on glyoxylate production from *cis*-aconitate. These data indicate the requirement for a metal cofactor; of the cations tested, magnesium most readily reversed the EDTA and pyrophosphate inhibitions.

Additional evidence for a metal requirement was obtained by using either dialysed Hughes-press extract (18 hr. dialysis against distilled water at 5°) or the fraction obtained by 30–60 % saturation with ammonium sulphate (preliminary experiments having indicated that the enzyme was precipitated by this treatment). Preparations made by either treatment had variable activity when mixed with substrate; sometimes glyoxylate was formed but on other occasions none was produced. In all cases, however, addition of the appropriate cofactor resulted in a greatly increased yield of glyoxylate. No glyoxylate was ever detected when either of these two types of enzyme

Table 3. *Formation of citrate from glyoxylate and succinate*

Experiments carried out in Warburg manometers. Each vessel contained 0·3 ml. of extract, 0·1 ml. of M tris buffer, pH 8·0; total vol., 2·0 ml.; incubation for 90 min. at 30° in $N_2$. Figures in parentheses show the amounts of substrate added in μmoles.

| Type of extract | Substrates added | Citrate formed (μmole) |
|---|---|---|
| Mickle | None | 0 |
| Mickle | Glyoxylate (10) +succinate (20) | 0·06 |
| Hughes press | None | 0 |
| Hughes press | Glyoxylate (10) | 0 |
| Hughes press | Succinate (20) | 0 |
| Hughes press | Glyoxylate (5) +succinate (20) | 0·56 |
| Hughes press | Glyoxylate (10) +succinate (20) | 0·68 |

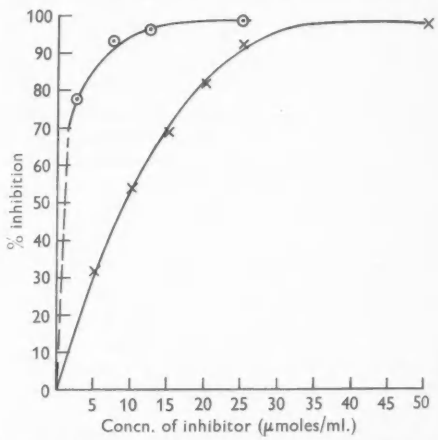



Fig. 3. Effect of EDTA and sodium pyrophosphate on formation of glyoxylate from *cis*-aconitate. Reactions carried out in Warburg manometers in $N_2$. Each vessel contained 0·1 ml. of M tris buffer, 30 μmoles of *cis*-aconitate; 0·5 ml. of Hughes-press extract of *Ps. aeruginosa*, amounts of EDTA and pyrophosphate as stated and water to 2 ml.; temp. 30°; duration of experiment 10 min. ⊙, EDTA; ×, pyrophosphate.

Table 4. *Cofactor requirements of enzyme system*

Experiments carried out in Warburg manometers. Flasks contained 0·3 ml. of enzyme preparation, 0·1 ml. of M tris buffer (pH 8·0), substrate (30 μmoles) and cofactor preparation (where added), 0·3 ml.; total vol. 2·0 ml.; incubation for 15 min. at 30° in $N_2$. BBY, boiled brewer's yeast extract.

| Enzyme preparation | Substrate | Cofactor | Glyoxylate formed (μmoles) |
|---|---|---|---|
| 30–60 % $(NH_4)_2SO_4$ fraction | None | BBY | 0 |
| | None | Ashed BBY | 0 |
| | None | Dialysed BBY | 0 |
| | *cis*-Aconitate | None | 0 |
| | *cis*-Aconitate | BBY | 3·78 |
| | *cis*-Aconitate | Ashed BBY | 2·57 |
| | *cis*-Aconitate | Dialysed BBY | 0 |
| | (+)-*iso*Citrate | None | 0 |
| | (+)-*iso*Citrate | BBY | 3·52 |
| | Citrate | None | 0 |
| | Citrate | BBY | 1·96 |
| Dialysed Hughes-press extract | *cis*-Aconitate | None | 0 |
| | *cis*-Aconitate | BBY | 2·1 |
| | *cis*-Aconitate | Ashed BBY | 0 |
| | *cis*-Aconitate | Dialysed BBY | 0 |

preparation was incubated in the absence of substrate. Table 4 gives some of the results obtained. In these particular experiments no glyoxylate was formed when either preparation was incubated with substrate alone. Addition to the 30–60% $(NH_4)_2SO_4$ fraction of a boiled extract of brewer's yeast caused glyoxylate formation from all three tricarboxylic acids, and this stimulating effect was prevented by dialysis of the BBY against distilled water at 5° for 18 hr. On the other hand, a solution of ashed BBY had some 67% of the activity of an equivalent amount of BBY. The activity of a dialysed Hughes-press extract was also stimulated by BBY but not by ashed or dialysed BBY.

*The substrate of the enzyme.* The preparations made by Campbell *et al.* (1953) had no action on *iso*citrate, but unfortunately the source of their *iso*citrate was not stated. Our crude extracts formed glyoxylate from all three tricarboxylic acids and, since there was a powerful aconitase present, it was impossible to decide which of these compounds was the substrate. It was found that both the 30–60% $(NH_4)_2SO_4$ fraction and a preparation obtained by heating the Hughes-press extract for 5 min. at 55° formed less glyoxylate from citrate than from either *cis*-aconitate or (+)-*iso*citrate.

It was also observed that the amount of glyoxalate formed from one sample of synthetic *iso*citrate was less than 25% of that produced from the naturally occurring compound (Table 5). Either there was an impurity in the synthetic material or one of the isomers inhibited the enzyme.

Table 5. *Comparison of activity on citrate,* iso*citrate and* cis*-aconitate*

Reaction carried out in Warburg manometers. Flasks contained 0·5 ml. of enzyme preparation from *Ps. aeruginosa*, 0·1 ml. of M tris buffer (pH 8·0), 30 μmoles of substrate; total vol. 2·0 ml.; incubation for 60 min. at 30° in $N_2$.

| Enzyme preparation | Substrate | Glyoxylate formed (μmoles) |
|---|---|---|
| Hughes-press extract, heated for 5 min. at 55° | Citrate | 1·35 |
| | *cis*-Aconitate | 2·57 |
| 30–60% $(NH_4)_2SO_4$ fraction from Hughes-press extract | Citrate | 1·35 |
| | *cis*-Aconitate | 5·4 |
| | (±)-*iso*Citrate | 1·15 |
| | (+)-*iso*Citrate | 5·7 |

## DISCUSSION

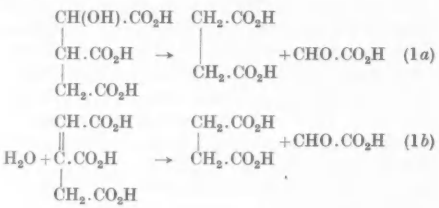
The experiments described show that untreated cell-free extracts of *Ps. aeruginosa* and *Ps. fluorescens* contain an enzyme system which converts the three tricarboxylic acids—citrate, *iso*citrate and *cis*-aconitate—into succinate and glyoxylate. In so far as these preparations attacked *iso*citrate our results differ from those reported by Campbell *et al.* (1953). These authors did not state whether they used natural or synthetic *iso*citrate, but if they used synthetic *iso*citrate their results could be readily explained by our observation that the enzyme is far less active with this substrate than with natural (+)-*iso*citrate.

In agreement with the observations of Campbell *et al.* (1953) and of Olson (1954), citrate is synthesized from glyoxylate and succinate. Since our preparations contained an active aconitase, no matter which of the three tricarboxylic acids is the actual product, citrate would be expected to accumulate. Only preparations made with the Hughes-press synthesized citrate from glyoxylate and succinate; extracts made with the Mickle disintegrator, while rapidly breaking down the three tricarboxylic acids, would not carry out the reverse reaction. This could be explained if either the succinate or the glyoxylate or both had to be activated and that only the Hughes-press extracts could form the active compound or compounds. The low yields of citrate observed may be due to the presence of only small amounts of the activating system in the preparation. In addition to a metal, an organic cofactor was essential. This was indicated by our experiments with dialysed preparations and with preparations obtained by fractionation with ammonium sulphate. Only the former were activated by boiled brewer's-yeast extract; the latter was activated either by the extract or by an ashed extract. Dialysis, it is believed, removed both the metal and the organic cofactor from the preparation; whereas precipitation with ammonium sulphate, while freeing the preparation of the metal, did not remove all the organic cofactor. A requirement for a metal was also indicated by the inhibitory action of EDTA and pyrophosphate when all three tricarboxylic acids were studied. The fact that these compounds were effective with all three substrates indicates that this inhibitory action was not exerted on aconitase.

The possibility that the reaction involves the conversion of citrate into oxaloacetate and acetate, followed by oxidation–reduction to give succinate and glyoxylate, may be excluded by the fact that preparations could be made which possessed less activity with citrate as substrate than with either *cis*-aconitate or *iso*citrate. It is perhaps also relevant that the direct oxidation of acetate to glyoxylate, although frequently postulated, has never been conclusively demonstrated.

It appears that either *iso*citrate or *cis*-aconitate is the substrate of this enzyme. If *iso*citrate is the substrate, the reaction can be pictured as analogous to that of the condensing enzyme. In

both cases the reaction involves the combination of a dicarboxylic acid with a monocarboxylic acid, one of which contains a carbonyl group. In the former case the carbonyl group is on the monocarboxylic acid; in the latter it is on the dicarboxylic acid. If *cis*-aconitate is the substrate, the reaction would involve the fission of a double bond by water. The two possibilities are illustrated by equations 1*a* and 1*b* respectively.




We prefer reaction 1*a*. Olson (1954), in his preliminary note, states that he has evidence supporting this idea.

That the treated extracts, while showing low activity against citrate, were about equally active with *iso*citrate and *cis*-aconitate could be interpreted to mean that the enzyme was able to utilize both substrates. However, the fact that these preparations had some slight activity with citrate indicates that a small amount of aconitase was present. The kinetics of aconitase with *cis*-aconitate as the substrate are such that a large amount is converted into *iso*citrate in the early stages of the reaction, although at equilibrium citrate is the major component (Krebs, 1950). Further, when aconitase acts upon citrate, there is a lag (Krebs & Holzach, 1952). Consequently if the substrate of this new enzyme is *iso*citrate, it is to be expected that preparations containing only a small amount of aconitase would be more active with *cis*-aconitate than with citrate.

The demonstration of the existence of this new enzyme system means that there is a second mechanism for the production of tricarboxylic acids and hence of α-oxoglutaric acid (cf. Krebs, Gurin & Eggleston, 1952). It also provides a cyclic mechanism for the oxidation of two-carbon compounds other than acetate, e.g. glycine and glycollate. Evidence has been accumulating that compounds such as these are oxidized by a mechanism which involves tricarboxylic acids. Thus Jayasuriya (1954), working with an oxalate-decomposing pseudomonad, found that washed suspensions of this organism, treated with monofluoroacetate, formed large amounts of citrate from glycine, glyoxylate or glycollate, and it would seem possible that the enzyme system described above is involved. Glyoxylate is known to be converted into glycine by animal tissues (see Weinhouse & Friedmann, 1951; Nakada & Weinhouse, 1953; Weissbach & Sprinson, 1953) and by plant tissues (Tolbert & Cohan, 1953; cf. Olson, 1954). Thus it is possible that this enzyme system participates in the biosynthesis of glycine by providing glyoxylate. The inter-relationships of this enzyme system with the tricarboxylic acid cycle are shown in Fig. 4.

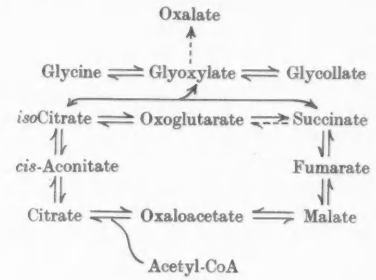



Fig. 4

Since this work was completed, Smith & Gunsalus (1954, 1955) have made preparations of this enzyme free from aconitase, and have found that only *iso*citrate is activated; they have also shown that magnesium ions and either glutathione or cysteine are essential cofactors; coenzyme A does not apprear to be involved.

## SUMMARY

1. Whole dried cells and cell-free extracts of *Pseudomonas aeruginosa* and *Ps. fluorescens* are capable of forming glyoxylate and succinate from citrate, *cis*-aconitate or *iso*citrate.

2. Glyoxylate was identified chromatographically as well as by isolation and melting-point determination of the 2:4-dinitrophenylhydrazone.

3. The reaction has been shown to be reversible. Crude extracts synthesize citrate from glyoxylate plus succinate.

4. Ethylenediaminetetraacetic acid and pyrophosphate strongly inhibit glyoxylate formation. Partially purified enzyme preparations appear to require both an inorganic and an organic cofactor.

5. Citrate was ruled out as a true substrate for the enzyme. Either *cis*-aconitate or natural (+)-*iso*citrate appears to be the substrate. Synthetic (±)-*iso*citrate may inhibit glyoxylate formation.

6. All results are consistent with the postulation that the $C_6$ substrate is cleaved directly to a $C_2$ and a $C_4$ acid.

The authors would like to express their gratitude to Dr S. R. Elsden and Professor H. A. Krebs, F.R.S., for their advice and encouragement throughout the course of

this work. The work was financed by the National Foundation for Infantile Paralysis, and by the Agricultural Research Council.

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# Bovine Serum Albumin and its Behaviour in Acid Solution

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(*Received* 26 *May* 1955)

In a recent communication Weber (1953*a*) suggested that the bovine serum albumin molecule dissociated at pH 1·86 into two sub-units of approximately equal molecular weight. This conclusion, although supported by other data, was based largely on measurements of the depolarization of fluorescence from solutions of protein-fluorescent dye conjugates, but it was strongly contested, particularly by Pedersen (1953). Since other workers, using light scattering (Doty & Steiner, 1952; Edsall, Edelhoch, Lontie & Morrison, 1950) or osmotic pressure (Gutfreund, 1954) have unequivocally demonstrated the constancy of the molecular weight from neutrality down to pH 3·3, it remains to investigate the pH range 3·3–1·8. In the present communication, a detailed examination by light-scattering and sedimentation velocity under these conditions has been undertaken, which with published data from other workers (Champagne & Sadron, 1954) shows clearly that no significant change in molecular weight occurs. [During the preparation of this manuscript, the paper by Reichmann & Charlwood (1954) appeared, in which, on the basis of light-scattering and sedimentation measurements, the same conclusion was reached.] A parallel investigation by depolarization of fluorescence, with other optical measurements necessary for the detailed interpretation of results, was also carried out.

As will be shown, the changes in the depolarization of fluorescence observed by Weber at acid pH values have, to a large extent, been confirmed and, in view of the constancy of the molecular weight of the protein, an explanation is of some importance. In particular, a decision as to whether the changes are due to definite internal changes (involving intramolecular rotations) in the protein molecule or to alteration in the fluorescence characteristics of the conjugating molecule is required. As to the latter possibility, the dimethylamino group of the fluorescent label (1-dimethylaminonaphthalene-5-sulphonamido) used by Weber and in some of the present work has a p$K$ value of about 4, and it is well known (Bowen & Wokes, 1953; Förster, 1951) that ionization may affect fluorescence behaviour. Accordingly, conjugates of bovine serum albumin with $\beta$-anthryl *iso*cyanate, a fluorescent non-ionizing compound used by Creech & Jones (1941), have been prepared and compared in behaviour with the naphthyl conjugates. In order to detect changes in fluorescence yield (which is related to the lifetime of the excited state), absorption and fluorescence spectra have been

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compared. It will be shown that, only if such auxiliary measurements can be made, is it feasible to apply the depolarization method over a range of experimental conditions, or in the investigation of an unknown macromolecule. For more complicated and reacting systems (Tsao, 1953), its application is much more hazardous.

## EXPERIMENTAL

'Armour' crystallized bovine serum albumin was used throughout this work. Concentrations were determined after dialysis against the solvent, by measuring interferometrically the refractive index difference $\Delta n$ between solution and solvent. A value of 0·1883 was taken for $dn/dc$ (Perlmann & Longsworth, 1948; Halwer, Nutting & Brice, 1951). Measurements of pH were made in a standard Cambridge pH meter, using a glass and calomel electrode assembly which was calibrated by 0·05 M potassium hydrogen phthalate at pH 3·97.

### *Preparation of conjugates*

*Preparation from 1-dimethylaminonaphthalene-5-sulphonyl chloride ('naphthyl' conjugates).*

The protein solution of concentration approx. 1%, in 1% $NaHCO_3$ solution, was allowed to react with 1–2% of its weight of 1-dimethylaminonaphthalene-5-sulphonyl chloride, dissolved in a small volume of A.R. acetone, in the manner described by Weber (1952*a*). The resultant conjugate solutions, after dialysis against 0·2 M-KCl to remove the main bulk of the free and adsorbed dyestuff, were then passed through an equilibrated column of basic ion-exchange resin (Dowex 2, mesh 200) to remove remaining traces. Conjugates were then tested for the absence of adsorbed material by an ascending paper chromatogram (Weber, 1953*a*). The absorption maximum of the conjugating group at about 3300 Å is sufficiently far removed from the protein absorption band to be unaffected by it; hence the number of conjugating groupings per molecule of protein was determined from the absorption at this wavelength, using for the molar extinction coefficient $\epsilon$ the value $4{\cdot}3 \times 10^6$ cm.²/mole (Weber, 1952*a*).

*Preparation of conjugates from β-anthryl isocyanate ('anthryl' conjugates).* These were prepared from β-anthryl *iso*cyanate (solution in A.R. dioxan) and bovine serum albumin (in 0·1 M-$Na_2HPO_4$), in the manner described by Creech & Jones (1941) for the conjugation of horse serum albumin. However, since only a lightly labelled conjugate was required (approx. 3 mol./mol. of protein), the amount of *iso*cyanate employed was reduced to about 20 mg./g. of protein. The resultant anthryl conjugates were freed of extraneous *iso*cyanate by two precipitations with 2·8 M-$(NH_4)_2SO_4$, followed by a final precipitation with acetone at $-18°$. The degree of conjugation was calculated from the absorption maximum of the β-anthryl carbamido-group which occurred at 3550 Å, using a value of $\epsilon$ of $3{\cdot}04 \times 10^6$ cm.²/mole, calculated from the value of $E^{1\%}_{1\,\mathrm{cm.}} = 139$, given by Creech & Jones.

### *Depolarization of fluorescence measurements*

Measurement of the degree of polarization of fluorescent radiation, over a temperature range 4–40°, was made in an apparatus similar to that described by Perrin (1929) and Weber (1952*b*), fluorescence being excited by means of a mercury arc and a Wood's glass filter. Observations of fluorescence depolarization were made at right angles to the direction of excitation, through an Ilford filter no. 110. We have found, however, that this polariscope, employing the method of observing the disappearance of fringes, was subjective and lacking in sensitivity. We have, therefore, adapted a polariscope of the type described by Wright (1934), which employs matching of fringes, a more sensitive and impartial procedure (W. F. Harrington, P. Johnson & R. H. Ottewill, unpublished).

It has been shown by Weber (1952*b*) that depolarization of the fluorescent radiation from conjugated proteins which are oblate or prolate ellipsoids of small asymmetry obeys a law analogous to that of Perrin (1926) for spherical molecules. For unpolarized exciting radiation, the equation may be written

$$\frac{1}{p}+\frac{1}{3}=\left(\frac{1}{p_0}+\frac{1}{3}\right)\left(1+3\frac{\tau_0}{\rho_h}\right), \qquad (1)$$

where $p$ represents the degree of polarization of fluorescent light emitted at right angles to the direction of the incident radiation, $\tau_0$ the lifetime of the excited state of the fluorescence, $\rho_h$ the harmonic mean of the two principal relaxation times of the rotation of the ellipsoidal molecule, and $p_0$ is an empirical constant. The value of $p_0$ may be determined experimentally by measuring the depolarization of the fluorescence of the conjugate in 60% (w/v) sucrose solution.

If the relation between $1/p$ and $T/\eta$ is linear ($T$ is the absolute temperature and $\eta$ the viscosity of the solvent) and $b$ is the slope of this curve, then, provided that the value of $\tau_0$ is not dependent on temperature, a quantity $\beta$ may be defined, where

$$\beta=\frac{b}{(1/p_0+\frac{1}{3})}=\frac{3\eta\tau_0}{\rho_h T}. \qquad (2)$$

The determination of $\rho_h$, over a range of temperature, requires ideally a comparable knowledge of the absolute value of $\tau_0$ for the conjugated protein, determined by a technique of the type described by Bailey & Rollefson (1953). This, however, is a problem of some magnitude and, of the other more approximate methods available, that devised by Lewis & Kasha (1945), or in an improved form by Förster (1951), may be employed. Thus $\tau_0$ can be derived from Förster's equation:

$$\frac{1}{\tau_0}=2{\cdot}88\times10^{-9}\,\frac{n^2\nu^2_{\max.}}{Q}\int_0^\infty \epsilon\nu\,\mathrm{d}\nu \text{ sec.}^{-1}, \qquad (3)$$

where $n$ represents the refractive index of the medium, $\nu_{\max.}$ the maximum of the absorption band in wave numbers, and $\int_0^\infty \epsilon\nu\,\mathrm{d}\nu\,(=A)$ the area of the absorption band. The fluorescence yield, $Q$, is defined as

$$\frac{\text{No. of fluorescent quanta emitted}}{\text{No. of incident quanta absorbed}}=\frac{kF}{A},$$

where $k$ is a constant and $F$ and $A$ are areas under the fluorescence and absorption bands respectively. Hence equation (3) may be written

$$\frac{1}{\tau_0}=\frac{K\nu^2_{\max.}A^2}{F}, \qquad (4)$$

where $K$ is a constant. It follows that

$$\frac{\tau}{\tau_0}=\frac{\nu^2_{0\max.}.A_0^2F}{\nu^2_{\max.}.A^2F_0}. \quad (5)$$

Thus if the value of $\tau_0$ can be well established in one environment, it is possible to estimate the value of $\tau$ in any other by a direct comparison of the absorption and fluorescence spectra in the two environments, measured under otherwise identical conditions of protein concentration and labelling.

Simpler cases result if (1) there is little or no shift in the absorption maximum between the two environments, giving

$$\frac{\tau}{\tau_0}=\frac{A_0^2F}{A^2F_0}, \quad (6)$$

and (2) the absorption characteristics are completely unchanged in both environments, when

$$\frac{\tau}{\tau_0}=\frac{F}{F_0}. \quad (7)$$

By accepting Weber's value of $\tau_0$ under neutral conditions (Weber, 1952*a*), the value of $\tau$ at acid pH may be obtained by a comparison of the fluorescence and absorption spectra (at concentrations below the range of concentration quenching) at neutral and acid pH. The value of $\rho_h$ may then be corrected by equation (2).

If, alternatively, measurements of the depolarization of the fluorescence of the conjugating molecule in a viscous solvent, e.g. glycerol, are substituted in the Perrin equation for unpolarized exciting radiation:

$$\frac{1}{p}+\frac{1}{3}=\left(\frac{1}{p_0}+\frac{1}{3}\right)\left(1+\frac{RT}{\eta V}\tau_0\right), \quad (8)$$

then $\tau_0$ may be obtained if some estimate can be made of the solvated volume $V$ of the fluorescent molecule in solution. This is a difficult estimation, and moreover the value of $\tau_0$ obtained is not necessarily that of the conjugating grouping when it is attached to the protein. Weber calculated the value of $\rho_h$ for bovine serum albumin and ovalbumin at the isoelectric points from the dielectric dispersion measurements of Oncley (1942), and, combining this with depolarization of fluorescence measurements, he found for naphthyl conjugates $\tau_0=1{\cdot}4\times10^{-8}$ sec. By employing this procedure for anthryl conjugates, a value of $\tau_0=4{\cdot}4\times10^{-8}$ sec. is obtained. Substitution in the Perrin equation gives $V=398$ ml., for $\beta$-anthryl *iso*cyanate in glycerol, a value which is in excess of the molecular volume in the solid state by a factor which is of the same order as for several other fluorescent substances studied by Marinesco (1927). The value of $\tau_0$ is of the same order as that for anthracene in acetone, $5\times10^{-8}$ sec., obtained by Rau (1949), using a fluorometer.

### *Absorption spectra and fluorimetry*

Absorption spectra were measured by a Unicam photoelectric quartz spectrophotometer type SP. 500.

Fluorescent intensity curves were obtained by using the fluorimeter attachment supplied for use with this instrument. The fluorescent standard employed was a solution of $1{\cdot}5\times10^{-5}$ g./ml. of 1-dimethylaminonaphthalene-5-sulphonic acid in dilute aqueous $NaHCO_3$. All solutions were filtered, before measurement, through a sintered-glass disk.

### *Light-scattering*

The measurements were carried out with the light-scattering apparatus described by Goring & Johnson (1952*a*), and, as incident light, the mercury green line 5461 Å was used. Solutions were clarified by ultrafiltration through collodion membranes prepared according to the directions of Goring & Johnson (1952*b*). Weight-average molecular weights, $M$, were calculated from the usual light-scattering equation for small symmetrical scatterers:

$$\frac{Hc}{\tau}=\frac{1}{M}+\frac{2bc}{RT^3}=\frac{1}{M}+2Bc, \quad (9)$$

where $c$ = concentration of solution (g./ml.), $H$ contains optical terms and $b$ ($=RTB$) is a constant from the osmotic pressure–concentration equation. For Rayleigh scatterers the turbidity $\tau$ is proportional to the scattering $I_{90}$,

$$\tau=CI_{90}, \quad (10)$$

where $C$ is a calibration factor, and for the apparatus used had the value $4{\cdot}35\times10^{-3}$ cm.$^{-1}$. All experiments were carried out at room temperature, $18\pm2°$.

### *Fluorescence spectra*

Since no specific apparatus for detailed investigation of fluorescence spectra was available, the light-scattering apparatus was modified for this purpose. Solutions were placed in the normal light-scattering cells, and fluorescence was excited by means of a mercury arc and a Wood's glass filter. By the use of a series of interference filters, with transmissions in the range 4000–6000 Å, in front of the electron multiplier, the intensity of fluorescence in the 90° position as a function of wavelength was investigated. The resultant spectra were then corrected for the spectral sensitivity of the photomultiplier and the transmission factor of the filters. Fluorescence intensities obtained in this manner have to be corrected for the polarization of the fluorescent emission, the appropriate equation being that given by Singleterry & Weinberger (1951):

$$I_{\text{av.}}=I_{90}\left(\frac{6-2p}{6-3p}\right), \quad (11)$$

where $I_{\text{av.}}$ is the intensity that would have been observed with complete depolarization of the emitted light. Solutions of the conjugated proteins were thus compared at neutral and acid pH but under otherwise identical conditions of protein and labelling dye concentration. In order to utilize equation 3, the product $I_{\text{av.}}\nu$ was plotted against $\nu$, giving what are henceforth termed modified fluorescence spectra.

The resolution obtained by this method is not high, but since no significant spectral displacements were involved, it was thought to be sufficiently good to indicate gross changes, and to be preferable to the photographic method with its multiplicity of corrections. However, for a more quantitative investigation of life-time changes, a more detailed study of the fluorescence spectra would be required.

### *Sedimentation*

Sedimentation-velocity experiments were carried out with a Phywe air-driven ultracentrifuge equipped with a Philpot diagonal schlieren optical system. The temperature rise of the rotor was recorded throughout each run by means of a thermistor placed 1 mm. above the rotor surface

some 3 cm. from the axis of rotation (P. Johnson, unpublished work). A correction factor was computed to reduce sedimentation constants to the viscosity and density of water at 20°.

*Optical rotation*

Optical-rotation measurements were made in a modified Schmidt & Haensch polarimeter. The polarimeter tube was maintained at the desired temperature (±0·2°) by pumping water from a thermostatically controlled bath through the outer jacket.

## RESULTS

*Naphthyl conjugates*

Depolarization of fluorescence measurements have been carried out on naphthyl conjugates of bovine serum albumin in phosphate buffer (pH 7·3, *I* 0·05), in distilled water adjusted to pH 1·9 with hydrochloric acid, in sodium chloride solutions of different ionic strengths at a pH of 1·9, and in 7 M urea solution (Fig. 1). In the latter case, in order to obtain a sufficient range of $T/\eta$ values, it was necessary to examine the depolarization of fluorescence at constant temperature (18°) in 7 M urea solutions containing varying proportions of sucrose. The dynamic viscosity of each solution was then determined from the relative kinematic viscosity and the density. To avoid quenching effects as far as possible, low degrees of labelling (less than 5 groups/protein molecule) and low protein concentrations (less than 0·1 g./100 ml.) were used, but within the range examined depolarization appeared to be independent of protein concentration.

The values of $p_0$ obtained by extrapolation with the solutions at pH 7·3, and in dilute hydrochloric acid at pH 1·9, were in good agreement with the values obtained by measurements of depolarization of fluorescence in 60% (w/v) sucrose. The plots in sodium chloride solutions are, however, slightly convex to the $T/\eta$ axis, an indication of an increasing degree of rotation with temperature. Assuming the value of $T/\eta$ for water at 25° to be $3{\cdot}33 \times 10^4$ (degrees/poise), and $\tau_0$ to be $1{\cdot}4 \times 10^{-8}$ sec., the $\rho_h$ values given in Table 1 were calculated. In the remainder of this paper, it will be assumed that $\rho_h$ values refer to water at 25°. Where the plots show curvature, the slopes towards their extremities are used in deducing the corresponding $\rho_h$ values.

The absorption spectra of the conjugating group on the protein at neutral and acid pH were determined by subtraction of the protein contribution (Fig. 2). It was found that no gross change in the absorption spectra of bovine serum albumin between pH 1·8 and neutrality occurs. With the fluorescence spectra (Fig. 3) no correction was necessary for the contribution of the protein, since the amount of scattering at the protein concentration employed (0·035%) was negligible compared with the fluorescence intensity. A small correction, by equation (11), was applied for the

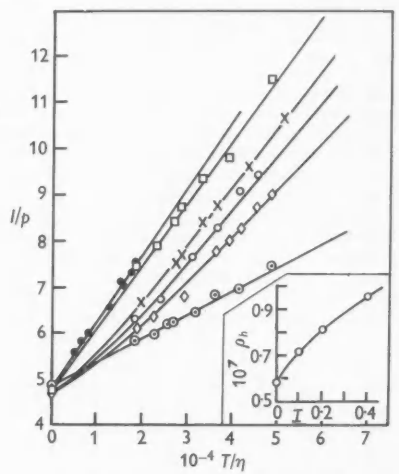


Fig. 1. Fluorescence polarization of naphthyl conjugates of bovine serum albumin (2·7 mol. of naphthyl derivative/mol. of protein). $1/p$ versus $T/\eta$ curves in: ●, 7 M urea solution; □, dilute hydrochloric acid, pH = 1·9; ×, dilute hydrochloric acid + 0·1 M-NaCl, pH = 1·9; ○, dilute hydrochloric acid + 0·2 M-NaCl, pH = 1·9; ◇, dilute hydrochloric acid + 0·4 M-NaCl, pH = 1·9; ⊙, phosphate buffer, pH 7·3, *I* 0·05. Protein concentrations of solutions, 0·02–0·08 g./100 ml.

*Inset.* Rotational relaxation time $\rho_h$ (corrected) at low $T/\eta$ values versus ionic strength.

Table 1. *Variation of rotational relaxation time* $\rho_h$ (25°) *with solvent for naphthyl conjugates (assuming* $\tau_0 = 1{\cdot}4 \times 10^{-8}$ *sec.)*

| Solution | pH | $1/p_0$ | $10^7 \rho_h$ (sec.) (±2%) High $T/\eta$ | Low $T/\eta$ |
|---|---|---|---|---|
| Dilute HCl | 1·90 | 4·76±0·05 | 0·47 | 0·47 |
| 0·1 M-NaCl + HCl | 1·97 | 4·59 | 0·47 | 0·58 |
| 0·2 M-NaCl + HCl | 1·92 | 4·60 | 0·46 | 0·66 |
| 0·4 M-NaCl + HCl | 1·92 | 4·60 | 0·59 | 0·78 |
| Phosphate buffer | 7·3 | 4·83 | 1·28 | 1·28 |
| 7 M urea | — | 4·80 | 0·45 | 0·45 |

polarization of the fluorescence. From the integrated areas of the absorption and emission curves, the values of $\tau$ in the different environments were calculated and the values of $\rho_h$ corrected accordingly (Table 2).

The value of $1{\cdot}28 \times 10^{-7}$ sec. for bovine serum albumin at pH 7·3, $I$ 0·05, is in good agreement with $1{\cdot}27 \times 10^{-7}$ sec. found experimentally by Weber (1952*a*), and $1{\cdot}24 \times 10^{-7}$ sec. calculated from the dielectric-dispersion measurements of Oncley (1942). The uncorrected value of $0{\cdot}47 \times 10^{-7}$ sec. found in dilute HCl at pH 1·9 is lower than Weber's (1953*a*) value (also uncorrected) of $0{\cdot}56 \times 10^{-7}$ sec., found under these conditions. After correction for life-time changes these values become $0{\cdot}58 \times 10^{-7}$ and $0{\cdot}69 \times 10^{-7}$ sec. respectively. On the addition of NaCl at pH 1·9 the rotational relaxation times at low $T/\eta$ values increase (Fig. 1, inset), but at higher $T/\eta$ values they approach $0{\cdot}58 \times 10^{-7}$ sec., except in 0·4 M-NaCl. In the latter calculations, the intercept on the $1/p$ axis of the linear plot at high $T/\eta$ values was used as the $1/p_0$ value. Thus though the changes in the lifetime of the excited state modify the apparent relaxation times in acid solution, they cannot in themselves wholly account for the reduction observed at pH 1·9. It is also of interest that in 7 M urea the $\rho_h$ value for bovine serum albumin is still further reduced to $0{\cdot}37 \times 10^{-7}$ sec., indicating slightly greater rotational freedom than in dilute HCl at pH 1·9.

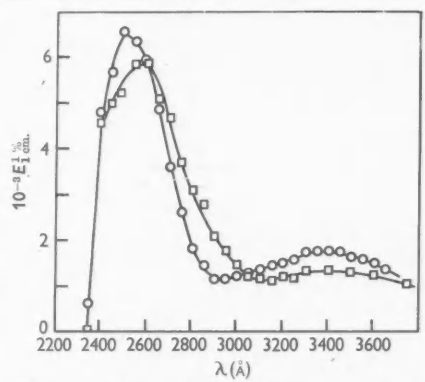


Fig. 2. Absorption spectra of the naphthyl grouping conjugated to the bovine serum albumin molecule (2·7 mol. of naphthyl derivative/mol. of protein) at: ○, pH 5·6; □, pH 2·0.

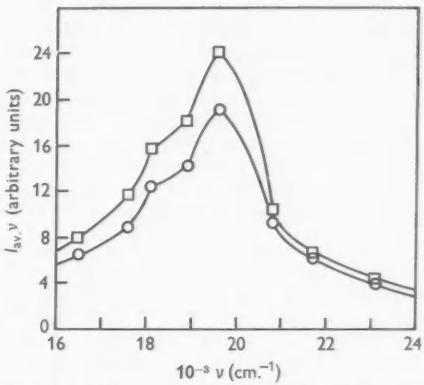


Fig. 3. Modified fluorescence spectra of naphthyl conjugates of bovine serum albumin (2·7 mol. of naphthyl derivative/mol. of protein) at: ○, pH = 5·6; □, pH = 2·0. Protein concentration, 0·035 g./100 ml.

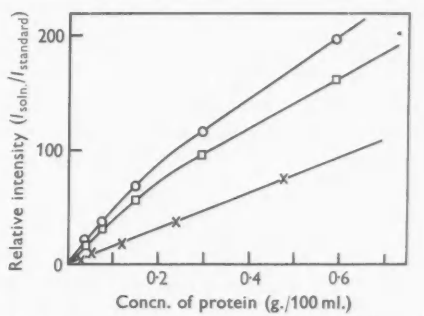


Fig. 4. Fluorescent intensity–protein concentration for naphthyl conjugates (4·9 mol. of naphthyl derivative/mol. of protein) in: ○, dilute hydrochloric acid at pH 1·9; □, distilled water at pH 5·3; and ×, 7 M urea.

Table 2. *Values of rotational relaxation time $\rho_h$ corrected for variation in the life time of the excited state (naphthyl conjugates)*

| Solution | pH | $10^8\,\tau$ (sec.) | $10^7\,\rho_h$ (sec.) (±2%) High $T/\eta$ | Low $T/\eta$ |
|---|---|---|---|---|
| Dilute HCl | 1·90 | 1·72 | 0·58 | 0·58 |
| 0·1 M-NaCl + HCl | 1·97 | 1·72 | 0·58 | 0·71 |
| 0·2 M-NaCl + HCl | 1·92 | 1·72 | 0·57 | 0·81 |
| 0·4 M-NaCl + HCl | 1·92 | 1·72 | 0·73 | 0·96 |
| Phosphate buffer | 7·3 | 1·40 | 1·28 | 1·28 |
| 7 M urea | — | 1·15 | 0·37 | 0·37 |

In Fig. 4 are shown the curves of relative total fluorescence intensity versus concentration of protein over a wide range of protein concentration for a sample containing 4·9 mol. of naphthyl derivative/mol. of protein. These demonstrate clearly the increase in fluorescent intensity that occurs with the naphthyl conjugate at pH 1·9 (cf. Fig. 3) in comparison with that under neutral conditions. With the free naphthylsulphonic acid, quenching is observed in the neighbourhood of pH 4. Weber (1953*b*) has attributed the lack of quenching of the conjugated protein to the strongly charged environment of the protein molecule. The quenching effect caused by 7 M urea solutions is also clearly shown in Fig. 4.

*Anthryl conjugates*

Studies on the depolarization of fluorescence have been carried out on anthryl conjugates, in phosphate buffer (pH 7·3, $I$ 0·05), and in dilute hydrochloric acid at pH 1·9. The curves obtained with these conjugates obey the linear law of depolarization (Fig. 5), but the polarization values obtained are smaller than those observed with naphthyl conjugates owing to the longer lifetime of the excited state ($4{\cdot}4 \times 10^{-8}$ sec. at pH 7·3). The values of $\rho_h$ obtained at pH 7·3 and 1·9 are 1·24 and $0{\cdot}80 \times 10^{-7}$ sec. respectively.

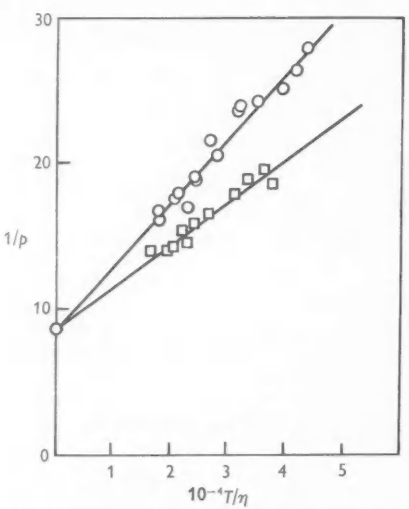


Fig. 5. Fluorescence polarization of anthryl conjugates of bovine serum albumin (3·9 mol. of anthryl derivative/mol. of protein), $1/p$ versus $T/\eta$ curves in: ○, dilute hydrochloric acid at pH 1·9; □, phosphate buffer, pH 7·3, $I$ 0·05. Protein concentrations approx. 0·02 g./100 ml.

To investigate possible changes in the lifetime of the excited state, the absorption and fluorescence spectra have been examined (Figs. 6, 7). A considerable increase in fluorescence intensity occurred at pH 1·9; this was confirmed by means of fluorimetric curves (Fig. 8), which also indicated that, in order to obtain data on the polarization of fluorescence on the linear portion of these curves, concentrations of about 0·02 % of conjugate had to be employed. At the same time, however, an increase

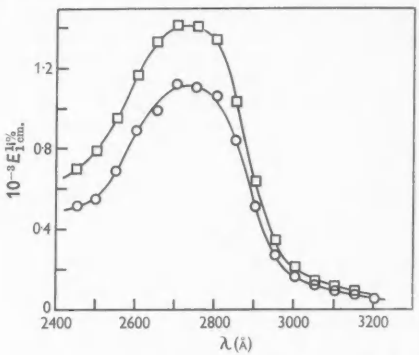


Fig. 6. Absorption spectra of the anthryl grouping conjugated to the bovine serum albumin molecule (3·9 mol. of anthryl derivative/mol. of protein). ○, pH 7·3, $I$ 0·05; □, pH 1·9.

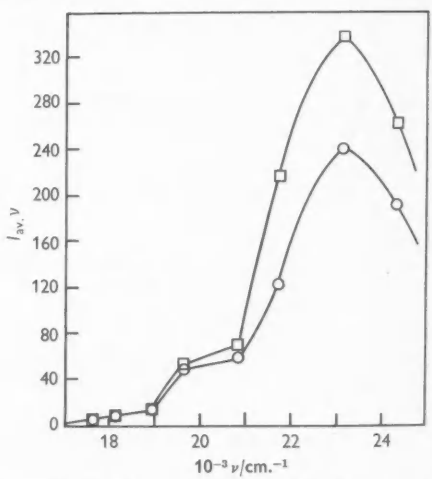


Fig. 7. Modified fluorescence spectra of anthryl conjugates of bovine serum albumin (3·9 mol. of anthryl derivative/mol. of protein) at: ○, pH 7·3, $I$ 0·05; □, pH 1·9. Protein concentration, 0·02 g./100 ml.

in absorption was observed, and, from the integrated areas of the fluorescent and absorption spectra, the lifetime of the excited state was calculated to be $3{\cdot}72 \times 10^{-8}$ sec. at pH 1·9, giving a corrected $\rho_h$ value of $0{\cdot}68 \times 10^{-7}$ sec.

Although somewhat different from the value of $0{\cdot}59 \times 10^{-7}$ sec. obtained for the naphthyl conjugates (compare the corrected value of Weber, $0{\cdot}69 \times 10^{-7}$ sec.), a difference which may possibly be accounted for by errors in the lifetime corrections, it is appreciably different from the value of $1{\cdot}28 \times 10^{-7}$ sec. obtained at neutral pH. It appears, therefore, that the major effects observed in bovine serum albumin conjugates (naphthyl or anthryl) by depolarization of fluorescence are not a function of the labelling group, and must to an appreciable extent be related to changes occurring in the protein molecule.

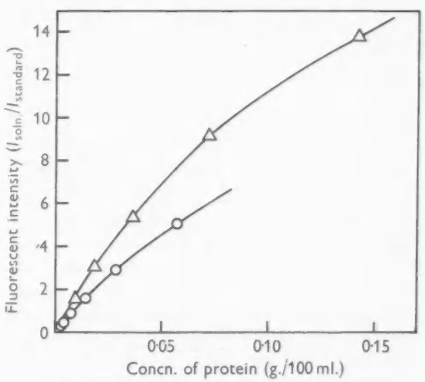


Fig. 8. Fluorescent intensity–protein concentration for anthryl conjugates (3·9 mol. of anthryl derivative/mol. of protein) in: △, dilute hydrochloric acid at pH 2·0; □, distilled water at pH 5·3.

*Light-scattering*

Light-scattering measurements have been carried out on solutions of bovine serum albumin at pH values of 4·5, 3·5, 2·3 and 1·86; the constitution of the solutions was as recorded in Table 3. Within experimental error all the curves gave good linear extrapolations to $(Hc/\tau)_{c=0} = 1{\cdot}39 \times 10^{-5}$ (Fig. 9), which gives a weight-average molecular weight of 72 000 ± 2000. The values of the gradient ($2B$), and the dissymmetries ($I_{60}/I_{120}$) of the most concentrated solutions at each ionic strength are recorded in Table 3. As stated elsewhere (Goring & Johnson, 1952*a*), variations in the dissymmetries, such as are observed, are not considered significant.

No aggregation effects of the type noticed by Goring (1952) were found in this work. It seems likely that the effects observed by him may have been due to a fatty acid impurity, occurring in some samples of bovine serum albumin, which is precipitated at acid pH (Yang & Foster, 1954). However, since the solutions in the present work were always ultrafiltered after adjustment of pH, it is unlikely that any effect of this type would have been encountered. It may therefore be concluded that no change of molecular weight occurs with bovine serum albumin in the pH range 4·5–1·9, at $I$ 0·10.

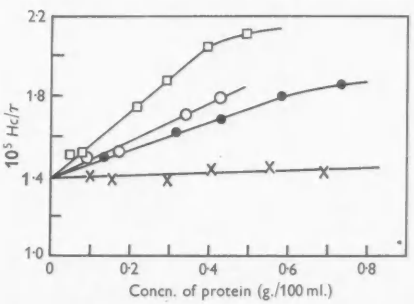


Fig. 9. Light-scattering curves of $Hc/\tau$ versus $c$ for bovine serum albumin in the pH range 1·86–4·5. □, pH 2·3, $I$ 0·065; ○, pH 1·86, $I$ 0·1; ●, pH 3·5, $I$ 0·1; ×, pH 4·5, $I$ 0·1.

*Optical rotation*

The optical rotation of solutions of bovine serum albumin in water and 0·1 M-NaCl to which sufficient HCl was added to give pH 1·9 was measured as a function of wavelength and temperature (Fig. 10). Linderstrøm-Lang & Schellman (1954) have shown that the wavelength variation may be expressed in the form

$$\frac{1}{[\alpha]} = \frac{\lambda^2}{A} - \frac{\lambda_c^2}{A}, \qquad (12)$$

Table 3. *Light-scattering functions for bovine serum albumin at acid* pH

| Solution | pH | Ionic strength | Dissymmetry ($I_{60}/I_{120}$) | $2B \times 10^5$ (eqn. 9) |
|---|---|---|---|---|
| HOAc + NaOAc + NaCl | 4·5 | 0·10 | 1·05 | 5·7 |
| HOAc + NaOAc + NaCl | 3·5 | 0·10 | 1·05 | 69·9 |
| HCl + glycine + NaCl | 2·3 | 0·07 | 1·02 | 165 |
| HCl + 0·1 M-NaCl | 1·9 | 0·11 | 1·09 | 93·9 |

where $A$ and $\lambda_c$ are empirical constants for a protein under given conditions; $\lambda_c$ seems to be a measure of the amount of secondary structure occurring in the protein. For native proteins it has a value 2450–2650 Å and for denatured materials it covers the range 2000–2300 Å. As Fig. 10 (*inset*) shows, $\lambda_c = 2650$ Å for bovine serum albumin at pH 1·9, somewhat larger than the value (2550 Å) given for the neutral protein. The occurrence of

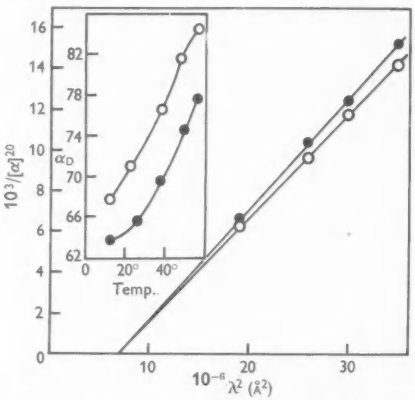


Fig. 10. Optical rotation measurements: $1/[\alpha]^{20}$ versus $\lambda^2$ for bovine serum albumin at pH 1·9 and protein concentration of 6·7 g./100 ml. ○, $l$ 0; ●, $l$ 0·1.
*Inset*. $[\alpha]_D$ versus temperature.

positive temperature coefficients of optical rotation has been found by Schellman (1955) to be associated with proteins having largely intact secondary structure, whereas denatured proteins invariably give a zero or negative temperature coefficient.

*Sedimentation velocity*

The sedimentation-velocity method was also used to investigate the effect of changes in ionic strength and pH on the molecule of bovine serum albumin. As a control the sedimentation coefficient as a function of protein concentration in 0·1 M-NaCl at pH 5·1 was determined. Fig. 11 summarizes these results and contains also the results of Creeth (1952) and of Kegeles & Gutter (1951), corrected in each case for the adiabatic cooling effect noted by Waugh & Yphantis (1952) and now accepted by many workers, e.g. Biancheria & Kegeles (1954). In each case a cooling of 1°, appropriate to a rotational speed of 60 000 rev./min., was assumed. A considerable measure of agreement between the different groups of results is apparent. The least-squares line for only the present results is given by the full line of Fig. 11, and its equation is

$$S^0_{20}\ (\text{Svedberg units}) = 4{\cdot}51(\pm 0{\cdot}05) - 0{\cdot}32(\pm 0{\cdot}11)\,c. \qquad (13)$$

Standard errors are given in parentheses. In evaluating their results, Kegeles & Gutter (1951) ignored sedimentation data at concentrations of 0·2 g./100 ml. and below on the grounds that, under such conditions, heavy impurities caused a spurious elevation of the sedimentation coefficient. In the present work no trend in the sedimentation

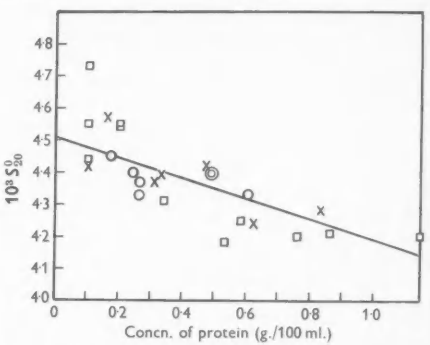


Fig. 11. Sedimentation coefficient $S^0_{20}$ versus protein concentration for bovine serum albumin. ×, Present work; □, Kegeles & Gutter (1951); ○, Creeth (1952).

coefficient during long runs was observed and all the results were used in calculating the constants of equation (13). Creeth, who also utilized data below 0·2 g./100 ml. concentration, obtained

$$S^0_{20} = 4{\cdot}42(\pm 0{\cdot}03) - 0{\cdot}12(\pm 0{\cdot}08)\,c \qquad (14)$$

to be compared with

$$S^0_{20} = 4{\cdot}38 - 0{\cdot}069c \qquad (15)$$

by Kegeles & Gutter. Although the $s$–$c$ slopes of the Spinco investigations are by no means in quantitative agreement, they are considerably smaller than that obtained in the present work, which is similar to that observed by Koenig & Pedersen (1950) over a wide concentration (up to 5% of protein) range. Sedimentation coefficients in the latter work are consistently at a higher level than those already quoted, but this would not be expected to affect the slope of the regression line. It is clear that the value for the slope of the $S^0_{20}$–$c$ line must remain uncertain until more precise results are available. For the sedimentation coefficient extrapolated to zero concentration $[S^0_{20}]$, the possible ranges of values given by the different recent workers are closely adjacent, and a rounded value of $[S^0_{20}] = 4{\cdot}45$S is to be recommended. It

seems highly unlikely, from the different experimental approaches supporting this value, that it can be in error by more than 0·1S.

To investigate the variation of sedimentation coefficient with pH at ionic strength, $I$ 0·1, a constant protein concentration of 0·32 g./100 ml. was used; at this concentration well-defined boundary peaks and sedimentation coefficients approaching those at infinite dilution are obtained. Fig. 12 summarizes the results obtained (with other physical measurements) and includes data from Svedberg & Sjögren (1930) obtained at 0·25% protein concentration. The fall in sedimentation coefficient as the pH is lowered below 4 is clearly shown by both sets of data, and, at a pH of about 2, the curve appears to flatten out at a value 3·43S.

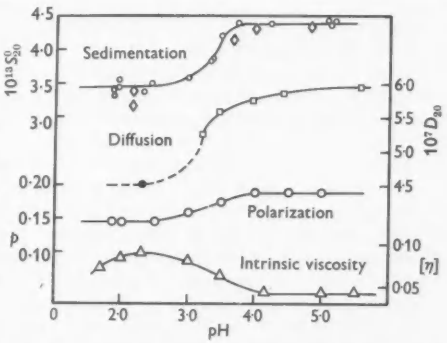


Fig. 12. Variation of sedimentation coefficient (○, $I$ 0·1, present work; ◇, $I$ 0·02–0·1, Svedberg & Sjögren, 1930), diffusion coefficient (□, $I$ 0·15, Champagne & Sadron, 1954; ●, $I$ 0·33, Kekwick, personal communication), polarization of fluorescent conjugates at $I$ 0·02 and 20° (Weber, 1952*a*), and intrinsic viscosity at $I$ 0·1 (Yang & Foster, 1954), with pH for bovine serum albumin.

The effect of changing ionic strength on sedimentation coefficient was investigated at constant protein concentration (0·32 g./100 ml.) and at two different levels of pH. Although at the higher pH (5·1) changes in ionic strength over the range 0·1–0·5 had no effect, at pH 1·90 pronounced effects were observed (Fig. 13). Thus below $I$ 0·3, reduction in ionic strength was accompanied by lowered sedimentation coefficient, an extrapolated value of 3·20S, corresponding to zero ionic strength, being obtained. Between $I$ 0·3 and 0·6 the sedimentation coefficient, 3·75S, was apparently independent of ionic strength. Above $I$ 0·6, some precipitation as well as aggregation of the protein occurred, demonstrated by the sedimentation diagrams of Fig. 14. It is to be noted that sedimentation coefficients of naphthyl and anthryl conjugates of bovine serum albumin fell, within experimental error, upon the curves for the unconjugated protein (Fig. 13), so that the presence of the conjugating agent does not affect the kinetic properties of the protein.

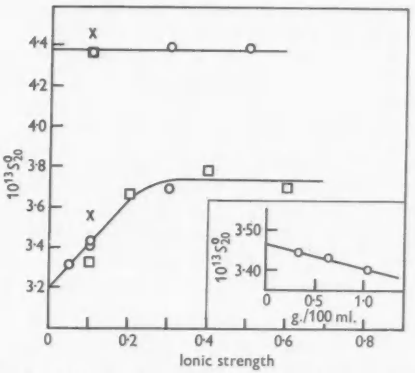


Fig. 13. Sedimentation coefficient, $S^0_{20}$–ionic strength for bovine serum albumin at pH 1·9 and 5·1. ○, Unconjugated bovine serum albumin; □, naphthyl conjugate; ×, anthryl conjugate. Protein concentration approx. 0·3 g./100 ml.

*Inset.* Sedimentation coefficient, $S^0_{20}$ versus protein concentration for bovine serum albumin at pH 1·9, $I$ 0·1.

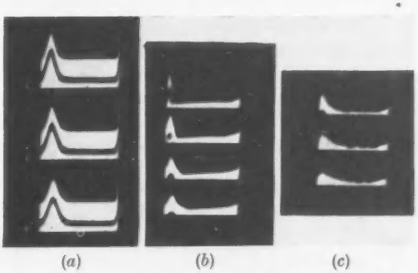


Fig. 14. Sedimentation diagrams for bovine serum albumin at pH 1·9 in different concentrations of sodium chloride. (*a*) 0·1 M-NaCl, 0·62% protein; (*b*) 0·6 M-NaCl, 0·32% protein; (*c*) 1·0 M-NaCl, 0·32% protein.

The curves of Fig. 13 refer to a protein concentration of 0·32 g./100 ml., and since the effect of acid conditions on the slope of the $S^0_{20}$–$c$ line was not known, a few runs at $I$ 0·1, pH 1·9, were carried out at different protein concentrations. The data shown in Fig. 13 (*inset*) are not sufficient to give the $S^0_{20}$–$c$ slope with accuracy, but the line drawn through the experimental results gives a slope which is quantitatively similar to that of Kegeles & Gutter under more neutral conditions. An $[S^0_{20}]$ value of 3·46S

was obtained by extrapolation, but it is less accurate than the isoelectric value. The partial specific volume determined pyknometrically at $I$ 0·1, pH 1·90 in NaCl–HCl solution at 20° was found to be $0{\cdot}729 \pm 0{\cdot}005$, in good agreement with that of Koenig (1950) under neutral conditions, but somewhat lower than that of Dayhoff, Perlmann & MacInnes (1952).

## DISCUSSION

### *Molecular weight and frictional ratio*

Diffusion coefficient measurements on bovine serum albumin have recently been performed by Creeth (1952), as well as by Champagne & Sadron (1954), somewhat differing results being reported. The latter authors obtained a value

$$5{\cdot}96(\pm 0{\cdot}02) \times 10^{-7} \text{ cm.}^2 \text{ sec.}^{-1},$$

independent of ionic strength (above $I$ 0·05) and protein concentration in the neighbourhood of the isoelectric point, but dependent on pH. Under similar conditions, Creeth obtained the value $6{\cdot}14\ (\pm 0{\cdot}02) \times 10^{-7}$, which was apparently independent of protein concentration and of small changes in ionic strength and pH. By combining these with the value $[S^0_{20}] = 4{\cdot}45$S already recommended we obtain the results of Table 4. The calculated molecular weight is rather critically dependent on the partial specific volume, but it seems clear that the molecular weight of the protein is in excess of the value (65 360) suggested by Creeth who used $\bar{v} = 0{\cdot}740$ as well as Spinco sedimentation coefficients uncorrected for temperature effects. The value $\bar{v} = 0{\cdot}734$ is probably accurate to $\pm 0{\cdot}002$, so that the molecular weight from sedimentation and diffusion may be given as $67\,500 \pm 1500$. Narrower limits await decisive information on the diffusion coefficient.

Recalculation of recent osmotic data (Gutfreund, 1954) also indicates a molecular weight value in good agreement with the last estimate. Thus Gutfreund used a $dn/dc$ value of 0·192, which is higher than is now accepted for bovine serum albumin in dilute salt solutions at $\lambda$ 5600 Å (utilized in interferometry). Using $dn/dc = 0{\cdot}1883$ (see page 570), we now obtain the molecular weight $67\,400 \pm 1000$. Light-scattering consistently gives molecular-weight values for bovine serum albumin of 70 000 and above (Edsall *et al.* 1950; Halwer *et al.* 1951; Goring & Johnson, 1952*a*), but in view of its weight-average character it would not appear to be a suitable method for accurate evaluation of molecular weight.

Fig. 12 contains the diffusion data of Champagne & Sadron (1954) and of R. A. Kekwick (personal communication), and the effect of pH on the diffusion coefficient is clearly very similar to that on the sedimentation coefficient. The molecular weights of Table 5 have been calculated by combining the diffusion and sedimentation data and assuming $\bar{v}$ to be 0·734. The sedimentation coefficients quoted strictly refer to 0·32 % of protein, but, for molecular-weight calculation, a slope of $-0{\cdot}20$ (i.e. $S = [S^0] - 0{\cdot}2c$) for the $s$–$c$ curve, within the range reported by various workers, has been assumed for correction purposes. The frictional ratios of Table 5 were calculated by assuming a constant value 67 500 for the molecular weight and the diffusion coefficients of column 2.

Allowing for the approximations used, it is clear that there is no change in the state of aggregation of the protein at $I$ 0·1 down to pH 2·3. Further, though light-scattering molecular weights are

Table 4. *Molecular weight of bovine serum albumin from sedimentation velocity and diffusion*

$$[S^0_{20}] = 4{\cdot}45\text{S}.$$

| Diffusion coeff. ($D_{20}$) (cm.$^2$ sec.$^{-1}$) | $\bar{v} = 0{\cdot}734$* | $\bar{v} = 0{\cdot}740$† |
|---|---|---|
| $5{\cdot}96 \times 10^{-7}$‡ | 68 200 | 69 800 |
| $6{\cdot}14 \times 10^{-7}$§ | 66 200 | 67 700 |

* Dayhoff, Perlmann & MacInnes (1952).
† Value used by Creeth and attributed to Pedersen (1945). The latter, however, gives $\bar{v} = 0{\cdot}736$.
‡ Champagne & Sadron (1954).
§ Creeth (1952).

Table 5. *Molecular weight and frictional ratio of bovine serum albumin as a function of* pH

0·1 M-NaCl. pH adjusted with HCl.

| pH | $10^7 D_{20}$ (cm.$^2$ sec.$^{-1}$) | $S^0_{20}$ (Svedberg units) | Mol.wt. | Frictional ratio $f/f_0$ |
|---|---|---|---|---|
| 5·3 | 6·0* | 4·42 | 68 200 | 1·31 |
| 4·4 | 5·85* | 4·41 | 69 800 | 1·35 |
| 3·95 | 5·74* | 4·39 | 70 800 | 1·37 |
| 3·45 | 5·58* | 4·05 | 67 250 | 1·41 |
| 3·2 | 5·25* | 3·75 | 66 250 | 1·50 |
| 2·3 | 4·49† | 3·43 | 71 000 | 1·75 |
| | | | Mean 68 900 | |

* From Champagne & Sadron (1954). † From Kekwick (personal communication).

somewhat higher, they confirm this conclusion and extend the pH-stability range to 1·9; no diffusion data are available at this value. Weber (1953*a*) quotes a sedimentation coefficient at pH 1·82 in 0·2 M-KCl of 2·7S (determined by Dr R. A. Kekwick) and a diffusion coefficient of $6{\cdot}85 \times 10^{-7}$. However, neither value is consistent with the more comprehensive data of Fig. 13. Dr R. A. Kekwick (personal communication) states that the sedimentation coefficient was obtained from one run only at 1% protein and that further experiments at 0·4% protein and pH 2·3 in phosphate buffer

$$(0{\cdot}124\,\text{M-}H_3PO_4 + 0{\cdot}206\,\text{M-}KH_2PO_4)$$

gave $S^0_{20} = 3{\cdot}68S$ (in fair agreement with the curve of Fig. 13 at pH 1·9) and $D_{20} = 4{\cdot}49 \times 10^{-7}$. The latter values yield a molecular weight of 74 000 for the preparation used.

Although the molecular weight does not vary as the pH is reduced to 1·9, the frictional ratio increases steadily. This behaviour is clearly reflected (see Fig. 12) in the curves against pH of intrinsic viscosity (drawn from the work of Yang & Foster, 1954) and of polarization of fluorescence of naphthyl conjugates of bovine serum albumin (see below). Yang & Foster (1954) also showed that significant changes in optical rotation occurred over the same pH range. Similarly, Gutfreund & Sturtevant (1953) detected a reversible first-order heat change (of 3100 cal.) in solutions of bovine serum albumin between pH 4·5 and 3·5. The occurrence of changes in sedimentation coefficient, diffusion coefficient, intrinsic viscosity, optical rotation, polarization of fluorescence and heat content over the pH range 4·5–2 strongly suggests a common explanation, and it seems likely that the changes are connected in some way with the increasing positive charge deriving from the titration of the carboxyl groups.

*Treatment in terms of the β function*

To investigate the meaning of the change in $f/f_0$, the approach of Scheraga & Mandelkern (1953) has been explored. Alternative suggestions made by Ogston (1953) have not been used, in view of their dependence on the $1/S$–$c$ slope. Applying in the first place to bovine serum albumin in neutral solution and using $[S^0_{20}] = 4{\cdot}45S$, $[\eta] = 0{\cdot}038$, $M = 67\,500$, $\bar{v} = 0{\cdot}734$, we obtain $\beta = 2{\cdot}05 \times 10^6$, a value lower than the permissible range of $\beta$ values. Tanford & Buzzell (1954), using slightly different data, obtained an even lower $\beta$ value. It seems unlikely that the data used here will be modified sufficiently to give $\beta \geqslant 2{\cdot}12 \times 10^6$, and an explanation of the lower observed value is of importance. Tanford & Buzzell suggested that departure from ellipsoidal form might be a possible explanation. In view of the straightforward use of the Simha viscosity equation and the Perrin expression (connecting frictional and axial ratios for the hydrodynamically equivalent ellipsoid of rotation) in determining the $\beta$ term, an alternative possibility would be that one (or both equations) is not holding accurately for nearly spherical particles. In particular, for axial ratios smaller than 20, an approximate form of the Simha equation has to be used. For the protein at $I$ 0·1, pH 1·9, we use $[S^0_{20}] = 3{\cdot}46S$, $[\eta] = 0{\cdot}097$ (from Yang & Foster, 1954) and other data as for the neutral protein, which gives $\beta = 2{\cdot}18 \times 10^6$. With normal interpretation this corresponds to an effective elongated ellipsoid of revolution of axial ratio 3·5. The corresponding values of $V_e$, the effective hydrodynamic ellipsoidal volume, at $I$ 0·1 under neutral and acidic (pH 1·9) conditions are 1·4 and $2{\cdot}7 \times 10^{-19}$ ml. In calculating the former, a shape factor, $\nu$, for viscosity of 3 was assumed. Both values are considerably in excess of

$$M\bar{v}/N = 0{\cdot}82 \times 10^{-19}\ \text{ml.},$$

and an expansion of the molecule at low pH seems clear. However, in view of the failure of the $\beta$-term treatment for the protein under neutral conditions, the results of these calculations are to be treated with caution. The data of Miller & Price (1946) for southern-bean mosaic virus, whose molecule appears accurately spherical from electron microscopy, give $\beta = 2{\cdot}3 \times 10^6$, which indicates an axial ratio of 7. On the other hand, recent results on tobacco mosaic virus, which from electron microscopy has a length:width ratio of about 19 in the unaggregated form, give $\beta = 2{\cdot}13 \times 10^6$ and an indicated axial ratio near unity. The data $[S^0_{20}] = 194S$ (W. F. Harrington & H. K. Schachman, unpublished results), $[\eta] = 0{\cdot}28$ (Schachman & Kauzmann, 1949), $\bar{v} = 0{\cdot}73$ (Schachman & Lauffer, 1949), and $M = 49 \times 10^6$ (Williams, Backus & Steere, 1951) were assumed. The effective ellipsoid given by the $\beta$ treatment thus appears to deviate substantially from the particles as they are indicated by electron microscopy, and the difference seems much larger than could be accounted for in terms of bound water.

If 30% hydration of bovine serum albumin is assumed, then the frictional ratios of Table 5 give prolate axial ratios of approximately 4 and 9 for the protein at $I$ 0·1 under neutral and acid (pH 2·3) conditions respectively. At pH 1·9 and lower ionic strengths, even higher frictional ratios occur, a limiting value for zero ionic strength of 1·9 being approached. However, the possible occurrence of primary charge effects at these low ionic strengths makes such extrapolation uncertain. On either derivation of molecular asymmetry, elongation of the molecule occurs at $I$ 0·1 as the pH is lowered and, though the use of higher ionic strengths partially reverses the elongation (as shown by the

$S^0_{20}$–ionic strength curve of Fig. 13, the limiting frictional ratio at $I \geqslant 0{\cdot}035$ has the value 1·62, considerably in excess of that under neutral conditions. Similar incomplete reversals in viscosity and optical rotation with increasing ionic strength were observed by Yang & Foster (1954). Even so, there are grounds for considering the change to be completely reversed on returning the pH to near neutrality. In particular, J. R. Marrack (personal communication) showed that bovine serum albumin exposed to pH 2 for 1 hr. gave a normal precipitin reaction after neutralization, and Yang & Foster (1954) showed the complete reversal, with increasing pH, of intrinsic viscosity, optical rotation and the binding of anionic detergent. Thus the limited nature of the changes in the protein at pH 1·9 is indicated, a view which is substantiated by the typically native $\lambda_c$ value obtained under these conditions from optical-rotation measurements. The high temperature coefficient at pH 1·9 is further evidence for the retention of appreciable secondary structure.

*Interpretation of polarization data*

Against this background of knowledge of the molecule of bovine serum albumin derived from largely established techniques, it is now possible to attempt an interpretation of the polarization data. In the first place the uncorrected values of $\rho_h$ obtained in this work both in neutral solution and at pH 1·9 are in reasonable agreement with those of Weber (1953*a*). Thus only his interpretation of results is questioned.

Further when Weber's fluorescent label has been replaced by a completely different structure ($\beta$-anthrylcarbamido-) and suitable correction (on the basis of absorption and fluorescence spectral measurements) for variation in the lifetime of the excited state has been applied, a similar reduction in $\rho_h$ is observed in acid solution and the actual $\rho_h$ values observed are in reasonable agreement with those (corrected) for the naphthyl conjugates. Nor is there any theoretical reason for considering that with lowering of pH similar fluorescence changes would occur for the two unrelated conjugating groups and thus account for depolarization results. The absence of such changes with naphthyl conjugates of ovalbumin again directs attention to possible changes in the protein part of the bovine serum albumin conjugate. The actual $\rho_h$ changes cannot be directly attributed to the increasing molecular asymmetry demonstrated already by sedimentation, diffusion and viscosity measurements, for an increase in relaxation time would thus be expected. Rather it would seem that additional rotational degrees of freedom become available at acid pH values, probably as a result of the high positive charge on the molecule and its consequent swelling. An isotropic swelling was suggested by Yang & Foster, but, as already shown, this is not in accord with physical measurements.

Consideration of some of the established properties of the bovine serum albumin molecule is relevant when the question of intramolecular rotation is discussed. Thus Kharush's (1950) suggestion of 'configurational adaptability' of the molecule, based upon the versatility of its binding properties, and the rapid and easy reversal of denaturation changes in the presence of urea, demonstrated by Kauzmann & Simpson (1953), Simpson & Kauzmann (1953) and Frendsdorf, Watson & Kauzmann (1953), are indications of 'looseness' of structure in the native protein, by which considerable alterations in internal structure would be expected as a result of somewhat minor changes in environment. The high optical rotation ($[\alpha]_D - 60°$) of bovine serum albumin compared with that of most native proteins ($[\alpha]_D - 20°$ to $-40°$) may well be attributable to incompleteness in internal hydrogen bonding which could allow such structural looseness. Thus the occurrence of intramolecular rotation when the equilibrium configuration of the native molecule of bovine serum albumin is disturbed by additional forces arising from the titration of the carboxyl groups seems reasonable. In ovalbumin and other proteins, further intramolecular rotations become possible only when much more drastic alteration of the original structure has occurred.

A decision on the type of rotation which becomes free at acid pH values is not easy. However, certain possibilities are worth mentioning. The limiting relaxation time observed at pH 1·9 ($0{\cdot}60 \times 10^{-7}$ sec.) corresponds with that of a free, compact and reasonably symmetrical molecule of molecular weight 35 000. Weber (1953*b*) has shown that when such units become flexibly joined the change in the observed relaxation time may be slight. Thus it seems possible that equal subunits may occur in the molecule of bovine serum albumin, which under neutral conditions are held by secondary forces so that their relative rotation is not possible. With increasing swelling and distortion of the molecule, such units would be freed, their relative rotation would be possible and their 'free' relaxation times would be observed, without necessarily affecting severely the internal structure of the subunits. A similar explanation seems very likely for fumarase in 0·1 M thiocyanate solution, where the relaxation time observed before dissociation occurs corresponds with that of the dissociation product (P. Johnson & V. Massey, unpublished).

It must be remembered that the molecule of bovine serum albumin has 16–18 intramolecular

cystine residues (Edsall, 1947; Hughes, Saroff & Carney, 1949), which considerably restrain the unfolding of the polypeptide chains. Thus the essential structure within each subunit could be retained by the presence of intramolecular disulphide cross-linkages, but free rotation would be possible about a short peptide segment connecting the two units. Such a hypothesis would be in keeping with the physical properties of bovine serum albumin described, and would explain the low relaxation time observed in acid solution. However, though such a model is likely to represent the truth, other possibilities exist. It would be difficult to rule out completely the freeing at acid pH values of segments of the polypeptide chain whose rotations might suffer various degrees of hindering. However, no reduction in $\rho_h$ for ovalbumin at acid pH appears to occur (Weber, 1952*a*). On either view, with increasing suppression of the electrostatic repulsions at higher ionic strengths, various degrees of hindering would occur, resulting in larger $\rho_h$ values. The opposite trend with increasing temperature is readily explained in terms of increased freedom of rotation with increasing thermal energy.

The indication from optical-rotation measurements of the 'native' type of structure in the molecule of bovine serum albumin at acid pH values is of considerable interest in view of the similar though lower relaxation time occurring in 7M urea solution, where few hydrogen bonds can remain. Presumably the stabilizing effect of the S–S bridges, limiting the extent of the structural changes, is responsible for this, as it is for the easy (though not necessarily complete) reversal of denaturation by urea and by acids. This view would be in keeping with either the subunit or the more general interpretation of the polarization changes outlined above.

## SUMMARY

1. New measurements of the depolarization of the fluorescence of conjugates of bovine serum albumin with fluorescent dyestuffs have been carried out over a range of pH and ionic strengths. Parallel investigations of the light absorption of the conjugating molecule, of the total fluorescence intensity and of the fluorescence spectra were made in order to detect changes in the life time of fluorescence. When such changes are allowed for, a decrease in the rotational relaxation time with decrease in pH below 4 was confirmed.

2. Light-scattering measurements have shown that, down to pH 1·9, over a range of ionic strengths, no change in molecular weight occurs. Sedimentation velocity measurements, with diffusion data already published, confirm this conclusion, but also indicate a swelling and increasing asymmetry of the molecule as the pH is lowered from 4 to 2. Increase in the ionic strength at constant pH only partially reverses such processes. Changes in viscosity, optical rotation and heat content of the solution occur over a similar pH range.

3. The decrease in rotational relaxation time is not directly connected with the increased molecular asymmetry at acid pH values, but must be attributed to increasing freedom of rotational motion within the molecule. Possible models in explanation of the observations are suggested.

4. New sedimentation data for the protein under neutral conditions are reported, and a molecular weight of 67 500, in good agreement with estimates from other methods, is suggested.

We are very grateful to the following: Professor H. J. Creech, for a gift of β-anthryl *iso*cyanate; Professor J. R. Marrack, for carrying out an immunological experiment; Dr G. Weber, for many helpful discussions over a considerable period of time; Professor K. Linderstrøm-Lang, for making available a polarimeter to one of us (W.F.H.); and Mr N. Buttress, for invaluable technical assistance. We are indebted to Dr G. Schellman for allowing us to read his manuscript (Schellman, 1955) before publication, and for many valuable discussions. Our thanks are also due to the Nuffield Foundation for a grant to one of us (R.H.O.).

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# Cholinesterase Inactivation by Snake Venoms

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(*Received* 23 *July* 1955)

Although the instability of the cholinesterase of cobra venom in aqueous solution has been pointed out by previous authors (Mounter, 1951; Augustinsson & Grahn, 1952), its cause is unknown. During studies of the cholinesterase of Formosan snake venoms, a high potency of cholinesterase inactivation (anticholinesterase) was found in the cholinesterase-containing venom of the Formosan cobra (*Naja naja atra*) (Chang & Lee, 1955). The existence of such anticholinesterase activity was proved by adding the cobra venom to the venom of *Bungarus multicinctus*, a very potent source of specific (true, acetyl-, aceto-) cholinesterase. Furthermore, in the presence of $MgCl_2$ (0·04 M) its anticholinesterase activity was completely inhibited.

The present work was undertaken to throw more light on the nature of the anticholinesterase activity of the cobra venom and to ascertain whether it can affect cholinesterase preparations from other sources. The results were presented in abstract at the meeting of the American Association for the Advancement of Science, Berkeley, California, in December, 1954.

## EXPERIMENTAL

*Measurement of cholinesterase activity.* The electrometric-titration method described by Alles & Hawes (1940) was adopted in most experiments for the measurement of cholinesterase activity with the aid of a Beckman pH meter, model G, unless otherwise stated. To 14 ml. of 0·0032 M acetylcholine chloride in 0·04 M-$MgCl_2$, 1 ml. of test solution was added. The reaction mixture was kept constantly at 37° by circulating water from a water bath. The pH was kept at 7·4, and the mixture was titrated with 0·02 N-NaOH to neutralize the liberated acetic acid. Cholinesterase activity is expressed in ml. of 0·02 N-NaOH consumed during the first 5 min. of enzymic hydrolysis of substrate.

In some experiments, rates of hydrolysis were measured by the Warburg manometric technique (Ammon, 1933). Each flask contained a total volume of 3 ml., all solutions being made up in 0·03 M-$NaHCO_3$, and after attachment to the manometers the flasks were gassed for 5 min. with $N_2+CO_2$ (95:5, v/v). No other salt was added to the system, unless otherwise indicated. The cholinesterase activity is expressed in $b_{30}$ values, i.e. the amount of $CO_2$ in $\mu$l. evolved during 30 min. minus the corresponding value for non-enzymic hydrolysis.

*Venoms.* Formosan cobra (*Naja naja atra*) venom was used for the study of the anticholinesterase activity, and the venom of *Bungarus multicinctus* was selected as the source of cholinesterase in most experiments. Venoms of other genera, which displayed no cholinesterase activity, were also tested for comparison in some experiments. The dried venoms were dissolved in cold distilled water before each experiment and stored in the ice box until used.

*Cholinesterase preparations from tissues.* Cholinesterase preparations from erythrocytes, serum, heart muscle and brain of rabbits were prepared in the manner described by Augustinsson (1948).

## RESULTS

*Effect of cations on* Naja*-venom anticholinesterase activity.* It has been demonstrated previously (Chang & Lee, 1955) that $MgCl_2$ (0·04 M) inhibits the anticholinesterase activity so completely that a typical bell-shaped p*S*–activity curve can be obtained for the *Naja*-venom cholinesterase in the presence of the salt. In order to determine how other cations affect the anticholinesterase activity of *Naja* venom, the venom solutions (*Naja* venom alone or *Naja* venom and *Bungarus* venom) were incubated with various salt solutions of different concentrations for 20 min. at 37° and the remaining cholinesterase was measured (Tables 1 and 2). It must be pointed out that the $MgCl_2$ added to the enzyme solution (1 ml.) did not significantly change the concentration of this cation in the substrate solution (14 ml.) during the measurement of cholinesterase. It can readily be seen that all the cations tested inhibited the *Naja*-venom anticholinesterase, although the degrees of inhibition varied considerably. In general, bivalent ions were more active than univalent ions. Thus, 0·02 M-$MgCl_2$ or -$CaCl_2$ was sufficient for complete inhibition of the *Naja*-venom anticholinesterase, whereas with NaCl, KCl, or $NH_4Cl$ 0·1 M solutions were needed. Among the univalent ions $NH_4^+$ was more active than $Na^+$ or $K^+$. Higher concentrations of either of the cations not only inhibited the *Naja*-venom anticholinesterase completely but also increased the cholinesterase activity beyond the original values. A possible explanation of these results is given in the Discussion.

The inactivation of cholinesterase by the *Naja*-venom anticholinesterase was irreversible: $MgCl_2$ (0·04 M) did not restore the cholinesterase activity

Table 1. *Effect of cations on anticholinesterase activity of* Naja *venom*

*Naja* venom (200 $\mu$g./ml. final concentration in the incubation mixture) was incubated with various salts at 37° for 20 min. The remaining cholinesterase (ChE) from 1 ml. of the incubation mixture was measured and the anticholinesterase (antiChE) activity (%) was computed against the original *Naja*-venom ChE. The original ChE before incubation was 0·23 and after incubation in the absence of any salt it decreased to nil.

| Salt concn. | $MgCl_2$ | | $CaCl_2$ | | NaCl | | KCl | | $NH_4Cl$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| (M) | ChE | AntiChE (%) | ChE | AntiChE (%) | ChE | AntiChE (%) | ChE | AntiChE (%) | ChE | AntiChE (%) |
| 1·0 | — | — | — | — | 0·27 | −17 | 0·27 | −17 | — | — |
| 0·1 | 0·32 | −36 | 0·27 | −17 | 0·22 | 4·5 | 0·21 | 9 | 0·27 | −17 |
| 0·02 | 0·23 | 0 | 0·23 | 0 | 0·006 | 97 | 0·01 | 96 | 0·095 | 59 |
| 0·002 | 0 | 100 | 0 | 100 | — | — | — | — | — | — |

Table 2. *Effect of cations on anticholinesterase activity of* Naja *venom*

In 1 ml. of incubation mixture, 30 $\mu$g. of *Bungarus* venom and 30 $\mu$g. of *Naja* venom (which was pre-incubated at 37° for 10 min. to destroy its own cholinesterase) were contained. Original cholinesterase (ChE) activity of 30 $\mu$g. *Bungarus* venom before incubation was 0·36, which decreased to 0·27 when incubated alone without *Naja* venom in the absence of any salt. The latter value was taken as control and used for the calculation of *Naja*-venom anticholinesterase (antiChE) activity (%). It was found to be 85% when no cations were added.

| Salt concn. | $MgCl_2$ | | $CaCl_2$ | | NaCl | | KCl | | $NH_4Cl$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| (M) | ChE | AntiChE (%) | ChE | AntiChE (%) | ChE | AntiChE (%) | ChE | AntiChE (%) | ChE | AntiChE (%) |
| 1·0 | — | — | — | — | 0·43 | −59 | 0·46 | −70 | — | — |
| 0·1 | 0·44 | −63 | 0·42 | −56 | 0·39 | −44 | 0·42 | −56 | 0·45 | −67 |
| 0·02 | 0·37 | −37 | 0·35 | −30 | 0·04 | 85 | 0·05 | 81 | 0·28 | −4 |
| 0·002 | 0·04 | 85 | 0·04 | 85 | — | — | — | — | — | — |

after the enzyme had been inactivated by the *Naja*-venom anticholinesterase.

On the other hand, inhibition of the *Naja*-venom anticholinesterase by $MgCl_2$ (0·02 M) or KCl (0·1 M) was not irreversible, since the anticholinesterase activity could be restored by diluting the mixture of the *Naja* venom and metal ions to the degree that no inhibition occurred (Table 3).

*Effects of organic substances on the* Naja-*venom anticholinesterase activity.* There seemed to exist some parallelism between inhibition of the anticholinesterase activity of *Naja* venom and stabilization or activation of cholinesterase by various cations, so it was of interest to ascertain whether or not certain organic substances which have been proved to act as stabilizers (Mounter, 1951) or activators of cholinesterase (Nachmansohn & Lederer, 1939; Chaudhuri, 1950*a*, *b*) could inhibit the *Naja*-venom anticholinesterase. The results of such experiments are shown in Tables 4 and 5.

It will be seen that cysteine, reduced glutathione, sodium citrate and hydrocyanic acid at a concentration of 0·02 M, and gelatin, bovine serum albumin and gum acacia at a concentration of 0·05 % not only inhibited the *Naja*-venom anticholinesterase completely but also increased the cholinesterase activity beyond the original values.

The cholinesterase of *Naja* or *Bungarus* venoms, once inactivated by the *Naja* venom anticholinesterase after 20 min. of incubation, could not be reactivated by the addition of cysteine, reduced glutathione, citrate or gelatin.

*Effect of hydrogen-ion concentration on the anticholinesterase activity of* Naja *venom.* *Bungarus*-venom cholinesterase (100 μg./ml.) was pre-incubated with *Naja* venom (20 μg./ml.) at various pH values for 40 min. at 37° and the remaining cholinesterase was measured at pH 7·4.

Table 3. *Reversibility of* Naja-*venom anticholinesterase, inhibited by cations, after dilution*

600 μg./ml. of *Naja* venom (after destroying its cholinesterase (ChE) by heating at 37° for 10 min.) was pre-incubated with $MgCl_2$ (final concn. 0·02 M) or KCl (final concn. 0·1 M) for 20 min. Thereafter, the mixture was diluted 10 times and its anticholinesterase (antiChE) activity against *Bungarus* venom was measured under the same conditions as in Table 2. Original ChE of 30 μg. of *Bungarus* venom before incubation was 0·30, which decreased to 0·25 after incubation.

| Salt added | ChE activity | AntiChE activity (%) |
|---|---|---|
| None (control) | 0·03 | 88 |
| $MgCl_2$ | 0·02 | 92 |
| KCl | 0·01 | 96 |

Table 4. *Effect of various organic substances on* Naja-*venom anticholinesterase*

Experimental conditions were the same as in Table 1. ChE, cholinesterase.

| Reagent | Concn. | ChE (original) | ChE (after incubation) | AntiChE (%) |
|---|---|---|---|---|
| None | — | 0·23 | 0 | 100 |
| Cysteine | 0·02 M | 0·23 | 0·25 | −9 |
| Glutathione | 0·02 M | 0·23 | 0·30 | −30 |
| Citrate | 0·02 M | 0·23 | 0·30 | −30 |
| Hydrocyanic acid | 0·02 M | 0·20 | 0·20 | 0 |
| Gelatin | 0·05 % (w/v) | 0·22 | 0·25 | −14 |
| Bovine serum albumin | 0·05 % (w/v) | 0·22 | 0·25 | −14 |
| Gum acacia | 0·05 % (w/v) | 0·22 | 0·25 | −14 |

Table 5. *Effect of various organic substances on* Naja-*venom anticholinesterase*

Experimental conditions were the same as in Table 2.

| Reagent | Concn. | ChE control | ChE after incubation with *Naja* venom | AntiChE % |
|---|---|---|---|---|
| None | — | 0·28* | 0·03 | 89 |
| Cysteine | 0·02 M | 0·28 | 0·39 | −39 |
| Glutathione | 0·02 M | 0·28 | 0·44 | −57 |
| Citrate | 0·02 M | 0·28 | 0·43 | −54 |
| None | — | 0·24† | 0·03 | 88 |
| Hydrocyanic acid | 0·02 M | 0·24 | 0·32 | −33 |
| Gelatin | 0·05 % (w/v) | 0·24 | 0·40 | −67 |
| Bovine serum albumin | 0·05 % (w/v) | 0·24 | 0·40 | −67 |
| Gum acacia | 0·05 % (w/v) | 0·24 | 0·35 | −46 |

* Original value of *Bungarus*-venom cholinesterase (ChE) before incubation, 0·36.
† Original value of *Bungarus*-venom ChE before incubation, 0·29.

As shown in Fig. 1, the *Naja*-venom anticholinesterase had a broad range of optimum activity, between pH 6·0 and 8·5. It started to decline abruptly at pH 5·5 and lost its activity completely below pH 5·0; on the alkaline side it started to decline more gradually at pH 9·0 and lost its activity completely above pH 10.

The pH range for the anticholinesterase activity of *Naja* venom was similar to that for specific cholinesterase (Wilson & Bergmann, 1950; Chang & Lee, 1955).

*Effect of temperature on the* Naja*-venom anticholinesterase.* As shown in Table 6, the *Naja*-venom anticholinesterase was stable below 50° but was completely destroyed by heating at 60° for 10 min. The heat-treated *Naja* venom seemed to stabilize the *Bungarus*-venom cholinesterase slightly during incubation.

*Effect of dialysis on the* Naja*-venom anticholinesterase.* As 0·2% (w/v) solution of *Naja* venom was dialysed at 5° for 2 days against distilled water, in a cellophan membrane. It will be seen from Table 7 that more than 80% of the *Naja*-venom anticholinesterase was lost after dialysis, since the anticholinesterase of 100 $\mu$g. of the dialysed venom was found to be much less than that of 20 $\mu$g. of the undialysed venom.

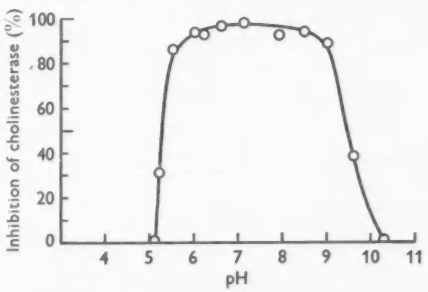


Fig. 1. Effect of hydrogen-ion concentration on the *Naja*-venom anticholinesterase activity.

*Relationship between anticholinesterase and proteolytic activity of various snake venoms.* In order to ascertain whether or not the anticholinesterase activity of *Naja* venom is due to a proteolytic enzyme in the venom, the two types of activity of various snake venoms were compared. As shown in Table 8, the proteolytic activity of the *Naja* venom was the least, whereas its anticholinesterase activity was the most potent among the venoms

Table 6. *Effect of temperature on stability of* Naja*-venom anticholinesterase*

1 mg./ml. of *Naja* venom was pre-incubated at the given temperature for 10 min. Thereafter, 30 $\mu$g. of the treated venom was incubated with 30 $\mu$g. of *Bungarus* venom in 1 ml. at 37° for 20 min. Cholinesterase (ChE) activity of 30 $\mu$g. of *Bungarus* venom alone after incubation for the same period was 0·23.

| Temperature (°) | Residual ChE | AntiChE activity (%) |
|---|---|---|
| 0 | 0·04 | 83 |
| 37 | 0·04 | 83 |
| 50 | 0·04 | 83 |
| 60 | 0·25 | −8 |
| 70 | 0·25 | −8 |
| 80 | 0·24 | −4 |
| 100 | 0·24 | −4 |

Table 7. *Effect of dialysis on* Naja*-venom anticholinesterase*

After dialysis, *Naja* venom was diluted and the anticholinesterase activities of 20 and 100 $\mu$g. were measured by incubation with 50 $\mu$g. of *Bungarus* venom in 1 ml. at 37° for 20 min.

| Dose of *Naja* venom ($\mu$g./ml.) | Anticholinesterase activity (%) | |
|---|---|---|
| | Without dialysis | After dialysis |
| 20 | 75 | 10 |
| 100 | 90 | 52 |

Table 8. *Relationship between anticholinesterase and proteolytic activities of various snake venoms*

| Source of venom | Proteolytic activity* | | Dose (mg.) | Anticholinesterase† | |
|---|---|---|---|---|---|
| | $MgCl_2$ absent | $MgCl_2$ (0·04 M) present | | $MgCl_2$ absent | $MgCl_2$ (0·04 M) present |
| *Naja naja atra* | 0·07 | 0·10 | 0·03 | 90 | −55 |
| *Trimeresurus mucrosquamatus* | 0·42 | 0·36 | 1·0 | 60 | −64 |
| *Trimeresurus gramineus* | 0·33 | — | 1·0 | 40 | −64 |
| *Agkistrodon acutus* | 0·33 | — | 1·0 | 5 | −60 |
| *Vipera russellii formosensis* | 0·11 | — | 1·0 | −20 | −64 |

* A 3·6% solution of casein was digested by venoms (2 mg. dry wt.; 1·0 mg./ml.) at pH 8·0, 40° for 1 hr. Activity is expressed as the difference in amounts (ml.) of 0·05 N ethanolic KOH needed to titrate 2·0 ml. of digestion mixture, which was brought to 90% ethanolic solution, before and after incubation.

† Venoms were incubated with 30 $\mu$g. of *Bungarus* venom in 1 ml. at 37° for 20 min. The pH of the incubation mixture was adjusted to 8·0–8·2. Anticholinesterase activity is expressed as a percentage of inhibition of the *Bungarus*-venom ChE.

tested. In general, no parallelism could be found between the two types of activity. Moreover, $MgCl_2$ (0·04 M) inhibited the anticholinesterase activity completely but scarcely affected the proteolytic activity.

From these experiments, it may be concluded that the anticholinesterase activity of *Naja* venom seems not to be due to a proteolytic enzyme.

*Inhibition of* Naja*-venom anticholinesterase by cholinesterase preparations from various tissues of rabbits.* *Naja*-venom anticholinesterase was easily inhibited by metal ions and other substances as mentioned above, and it remained to be determined whether cholinesterase preparations from mammalian tissues could be affected by the *Naja*-venom anticholinesterase.

As shown in Table 9, all the cholinesterase preparations tested (erythrocyte, serum, heart muscle and brain) not only failed to be inactivated by the at 100° for 10 min., whereas the cholinesterase of brain homogenate was destroyed by heating at 60° for the same period (Table 10).

## DISCUSSION

Chang & Lee (1955) have already suggested that the instability of the cholinesterase of cobra venom could be due to the coexistence of anticholinesterase activity, although the venom studied by them was a Formosan variety. Additional evidence for such a possibility is presented in the present work. Thus substances which stabilize the cholinesterase of cobra venom, such as metal ions (Augustinsson & Grahn, 1952), gelatin, gum acacia and albumin (Mounter, 1951), were shown to protect the cholinesterase from destruction by the *Naja*-venom anticholinesterase. Moreover, the order of the potency for the inhibition of anticholinesterase

Table 9. *Effect of* Naja*-venom anticholinesterase on cholinesterase from various tissues of rabbits*

Cholinesterase (ChE) activity was measured by the manometric method.

| | Cholinesterase activity ($b_{30}$) | | | | |
|---|---|---|---|---|---|
| Source of ChE | Control ($A$) | +venom (100 μg.) ($B$) | Difference ($B-A$) | +venom (200 μg.) ($C$) | Difference ($C-A$) |
| Erythrocyte | 9 | 82 | 73 | 146 | 137 |
| Serum | 26 | 102 | 76 | 156 | 130 |
| Heart muscle | 38 | 143 | 105 | 200 | 162 |
| Brain | 86 | 171 | 85 | 215 | 129 |
| Brain (dialysed) | 74 | 107 | 33 | 140 | 66 |

Table 10. *Thermostability of the factors inhibiting* Naja*-venom anticholinesterase in rabbit-brain homogenate*

Cholinesterase activity was measured by the manometric method.

| | Cholinesterase activity ($b_{30}$) | | | | |
|---|---|---|---|---|---|
| Temperature | Control ($A$) | +venom (100 μg.) ($B$) | Difference ($B-A$) | +venom (200 μg.) ($C$) | Difference ($C-A$) |
| Control (untreated) | 108 | 161 | 53 | 201 | 93 |
| 50° | 95 | 154 | 59 | 193 | 98 |
| 60° | 6 | 92 | 86 | 151 | 145 |
| 100° | 3 | 86 | 83 | 144 | 141 |

*Naja*-venom anticholinesterase, but on the contrary seemed to inhibit the latter activity. Consequently, the *Naja*-venom cholinesterase contributed its activity to that of the cholinesterase of the tissue preparations. It is apparent that cholinesterase preparations from various tissues of the rabbit contain some factor, or factors, which inhibit the *Naja*-venom anticholinesterase completely. Experiments with dialysed brain preparation (30 hr. against distilled water at 5°) indicated that the inhibiting factors were at least partially dialysable (Table 9). These factors were unaffected by heating

activity of various cations was found to be similar to their cholinesterase-stabilizing effects described by Augustinsson & Grahn. Some reducing agents, such as cysteine, reduced glutathione, hydrocyanic acid and citrate, which are activators of cholinesterase (Nachmansohn & Lederer, 1939; Chaudhuri, 1950*a*, *b*), were also found to be good inhibitors of the *Naja*-venom anticholinesterase. In addition to inhibiting the *Naja*-venom anticholinesterase, most of these compounds increased the cholinesterase activity beyond its original value. However, experiments with cations, especi-

ally with $MgCl_2$, showed that it is unlikely that their effect was due simply to activation of the cholinesterase, since there was sufficient $MgCl_2$ (0·04M) in the substrate solution during the measurement of cholinesterase. Therefore the increase of cholinesterase activity by pre-incubation with higher concentrations of cations must be due to some unknown mechanism other than simple activation of the enzyme. Since pre-incubation with the cations does not appear to be necessary for full activation of cholinesterase, it is assumed that the venoms contain some inert cholinesterase, which is loosely and reversibly bound to the anticholinesterase, and that the cations (possibly also the other inhibitors of the *Naja*-venom anticholinesterase) can reactivate the inert cholinesterase, but that reactivation is inhibited in the presence of substrate. On the basis of these assumptions, the experimental results can be readily explained. However, once the cholinesterase had been inactivated by incubation with the *Naja*-venom anticholinesterase, none of the inhibitors of the anticholinesterase could restore the cholinesterase activity.

On the other hand, the dilution experiments show that inhibition of the *Naja*-venom anticholinesterase by metal ions is not irreversible. In other words, metal ions do not destroy the *Naja*-venom anticholinesterase but only interfere with its interaction with cholinesterase.

The potent protection of cholinesterase, by the reducing agents tested, from destruction by the *Naja*-venom anticholinesterase might lead to an assumption that the anticholinesterase contains active disulphide groups and produces its effect by oxidizing the thiol groups of cholinesterase. However, this is improbable since the cholinesterase, once inactivated by the *Naja*-venom anticholinesterase, cannot be reactivated by these reducing agents. Moreover, the thiol group in cholinesterase was recently regarded not to be a significant group in the active surface of the enzyme (Mounter & Whittaker, 1953; Markwardt, 1953).

Since the pH range for the anticholinesterase activity of *Naja* venom is similar to that for the cholinesterase, it is suggested that the venom anticholinesterase inactivates the cholinesterase by attacking its active centre, just as does tetraethyl pyrophosphate (Wilson, 1954).

The dialysis experiments indicate that the *Naja*-venom anticholinesterase is not a compound of very high molecular weight, such as a protein, although the heating experiments suggested such a possibility. The possibility that the *Naja*-venom anticholinesterase is due to a proteolytic enzyme is unlikely, since no parallelism between two types of activity of various snake venoms could be found. Moreover, $MgCl_2$ inhibited the former activity but not the latter.

Finally, since cholinesterase preparations from various tissues contain some factor, or factors, which inhibit the *Naja*-venom anticholinesterase completely, they are unaffected by the venom anticholinesterase. These factors were found to be heat-resistant and at least partially dialysable.

## SUMMARY

1. The cholinesterase-inactivating activity (anticholinesterase) of the Formosan cobra venom (*Naja* venom) is reversibly inhibited by various cations. Magnesium is by far the most potent ion tested; sodium and potassium are least active in this respect.

2. Other activators or stabilizers of cholinesterase, such as cysteine, reduced glutathione, citrate, hydrocyanic acid, gelatin, albumin and gum acacia, are also inhibitors of the *Naja*-venom anticholinesterase.

3. The inactivation of cholinesterase by the *Naja*-venom anticholinesterase is irreversible, since, once the cholinesterase has been inactivated by the anticholinesterase, none of the anticholinesterase inhibitors can restore the cholinesterase activity.

4. In addition to the inhibition of the *Naja*-venom anticholinesterase, most of the inhibitors increase the cholinesterase activity beyond its original value. The possible cause is discussed.

5. The optimum pH range for the *Naja*-venom anticholinesterase is between 6·0 and 8·5. It loses its activity completely below 5·0 or above 10.

6. The *Naja*-venom anticholinesterase is completely destroyed by heating at 60° for 10 min. Its activity disappears for the most part after dialysis of a 0·2% solution.

7. The anticholinesterase activity of *Naja* venom seems not to be due to a proteolytic enzyme, since no parallelism between the two types of activity of various snake venoms could be found.

8. The *Naja*-venom anticholinesterase does not affect the cholinesterase preparations of various tissues of the rabbit such as erythrocyte, serum, heart muscle and brain, since they apparently contain some factors which inhibit the anticholinesterase completely. These factors are heat-resistant (100°, 10 min.) and at least partially dialysable.

The authors wish to thank Dr G. B. Koelle, Graduate School of Medicine, University of Pennsylvania, U.S.A., for his interest and advice in this work. This investigation was supported in part by a research grant [B-282 (C2)] from the National Institutes of Health, U.S. Public Health Service.

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# Purification and Amino Acid Analysis of Melanophore-expanding Hormone from Hog and Ox Pituitary Glands

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(*Received* 18 *July* 1955)

The preparation of highly purified, if not pure, melanophore-expanding hormone from hog pituitary has recently been reported by Lerner & Lee (1955). The present writers, with the aid of countercurrent distribution technique, have succeeded in preparing highly purified melanophore-expanding material, virtually free from adrenocorticotropic (ACTH) activity, from both hog and ox pituitary. The products from the two sources are not chemically identical. The amino acid composition of the material from hog pituitary differs considerably from that reported by Lerner & Lee (1955). The successful segregation of the melanophore-expanding principle and ACTH along with other evidence indicates the non-identity of the two hormones.

## EXPERIMENTAL

### *Preparation of material for countercurrent distribution*

(*a*) *Hog-pituitary extract.* A quantity (150 g.) of acetone-dried and powdered posterior pituitaries, assaying from 6 to 10 units of melanophore-hormone activity per mg., was divided into 10 g. lots and each lot was extracted with 25 ml. of acetone and 160 ml. of glacial acetic acid at 70°. The material was left in contact with the solvent at this temperature for 10 min. The extracts were centrifuged, the sediment of tissue fragments was removed and washed with 20 ml. each of cold acetone and glacial acetic acid and the washing was added to the previous extract. To the combined supernatant fluids were added 1 ml. of saturated NaCl and a quantity of acetone equal to half the volume of the extract. The resulting precipitate was removed by centrifuging and to the supernatant fluid was added an equal volume of diethyl ether. The precipitate was collected by centrifuging, washed three times with acetone and placed in a desiccator. The total yield of this partially purified material from 150 g. of posterior-pituitary lobes was 27 g. The melanophore-hormone activity was 30 units/mg., thus representing a recovery of 67·5% of the activity of the original material. Further purification of the product was achieved by dissolving 27 g. of the ether precipitate in 750 ml. of 0·1 N acetic acid. Oxycellulose (4·5 g.), previously washed successively with 1 N-HCl, water and 0·1 N acetic acid, was added and the mixture stirred for 40 hr. at room temperature. The oxycellulose was collected by centrifuging, washed successively with 0·1 N acetic acid and water, and then suspended twice in 100 ml. of 0·1 N-HCl and stirred for 30 min. to elute the hormone. The oxycellulose was removed by centrifuging and the pH of the combined supernatant fluids was adjusted to 6·5 with aq. N-$NH_3$. The resulting precipitate was removed by centrifuging and to the supernatant fluid 5 vol. of acetone were added. The resulting precipitate was washed with acetone and stored in an evacuated desiccator. The yield was 660 mg. of a powder which assayed 800 units/mg., representing a recovery of 44% of the activity of the original material. This procedure was based on the methods of Payne, Raben & Astwood (1950), and Astwood, Raben, Payne & Grady (1951), originally developed for the purification of ACTH, first used by Raben, Rosenberg & Astwood (1952) for the purification of melanophore hormone and later modified for this purpose by Landgrebe & Mitchell (1954). The present authors have introduced only slight modifications.

(*b*) *Ox-pituitary extract.* A quantity of purified powder which had been prepared from ox posterior pituitary lobes by Stehle (1944) was available. Stehle's method involved fractionation of an acetic acid extract of the tissue with organic solvents, and final isolation of the active material by precipitation with picric acid. The powder assayed 150 units/mg.

### *Countercurrent distribution*

For the segregation and purification of the melanophore-expanding substance and ACTH a 100-tube (each 200 ml. capacity) all-glass Craig apparatus was used. For analytical purposes a 24-unit (20 ml.) stainless-steel Craig instrument

was used. At the end of the countercurrent run the solvent mixture from each tube was homogenized by the addition of a small quantity of ethanol. The optical density of the solution at 2775 Å was determined in a Beckman DU spectrophotometer. This method has been used extensively for following the distribution of ACTH in counter-current extraction (Kuehl, Meisinger, Brink & Folkers, 1953). The solute was recovered by concentrating the solution, under reduced pressure in an atmosphere of nitrogen, to about 1 ml. volume, transferring the concentrate to a 100 ml. graduated cylinder with the aid of 4 ml. of methanol, and precipitating the solute by addition of 95 ml. of ethyl acetate. The precipitate was collected by centrifuging and dried in a desiccator.

### *Potentiation of the melanophore-expanding activity by treatment*

A sample (1 mg.) of the purified product was dissolved in 1 ml. of 0·1 N-NaOH and placed in a boiling-water bath for 15 min. The solution, cooled to room temperature, was neutralized to pH 7 with 0·1 N-HCl and assayed with appropriate dilution for melanophore-expanding potency.

### *Assay of melanophore-expanding activity*

The potency of the material, after appropriate dilution, was assayed in the intact frog (*Rana pipiens*). Each preparation was assayed three times on four frogs at various dose levels. The degree and the duration of the darkening of the skin colour were taken as criteria of the response. A small, known quantity of the solution, diluted to 1 ml. with 0·8% NaCl, was injected into the dorsal-lymph sac. A solution of the International Standard Posterior Pituitary Powder, prepared according to the stipulations of the British Pharmacopoeia, was used as the standard. In keeping with the definition of the unit of oxytocic and vasopressor activity, 1 mg. of this standard powder is considered to be equivalent to 2 i.u.

### *ACTH assay*

The assays for adrenocorticotropic potency of the fractions were performed by Saffran & Schally (1955), with their *in vitro* method. Before being assayed the material prepared from hog pituitary was treated with hydrogen sulphide to insure against loss of activity (Kuehl *et al.* 1953).

### *Determination of amino acid composition*

A small quantity (2–4 mg.) of the powder was dissolved in 0·2–0·4 ml. of 6 N-HCl in a 1 ml. ampoule. The ampoule was sealed and heated at 100° for 22 hr. The contents were evaporated *in vacuo* over solid sodium hydroxide and anhydrous calcium chloride. The residues were dissolved in sufficient water to give a concentration of 0·15 mg./0·02 ml. of solution. By the use of this quantity of the solution the amino acids were separated by paper chromatography as described in a previous publication (Benfey, 1953), with 0·5% ninhydrin in absolute ethanol as the indicator. After a period of 24 hr. for colour development, the stained areas were cut out and transferred to 15 ml. centrifuge tubes containing 7 ml. of 50% *n*-propanol to elute the colour. The optical density of the solution was determined at 5700 Å for all the amino acids except proline, which was read at 3500 Å. As standards for comparison solutions of pure amino acids in quantities equivalent to 0·07 μmole of amino acid were chromatographed. Three chromatographic runs were carried out with each solvent system, each unknown being accompanied by a standard mixture according to the method of Wellington (1952). With limited facilities this procedure affords satisfactory quantitative estimations of the amino acids. Tryptophan was determined according to the method of Spies & Chambers (1948).

## RESULTS

### *Hog-pituitary extract*

A plot of the spectrophotometric absorption of the contents of the individual units of the counter-current apparatus in our preliminary runs revealed two peaks. The fractions represented by one peak were found to contain the melanophore-expanding material and those corresponding to the other peak the adrenocorticotropic activity. When 660 mg. of the purified hog-pituitary powder was used, 180 transfers were applied in the large Craig apparatus with equal volumes (100 ml.) of 0·5% trichloroacetic acid and *sec.*-butanol as the solvent system. The units (tubes), 69–71 inclusive in this series, were found to contain the highest melanophore activity. The calculated partition ratio ($K$) of the contents was 0·64, and after removal of the solvents 16·2 mg. of material was obtained. The

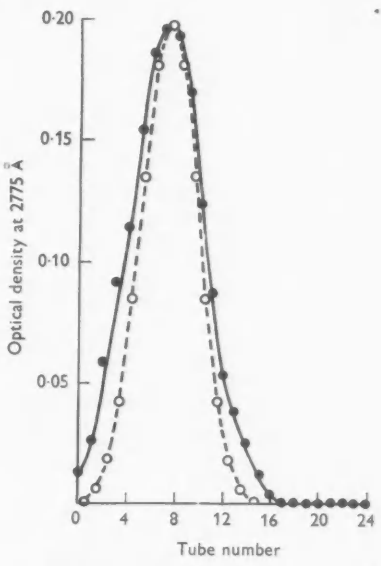


Fig. 1. Analytical redistribution of hog melanophore-expanding hormone between 0·5% trichloroacetic acid and *sec.*-butanol. ●, Experimental; ○, calculated.

melanophore-expanding activity of the product was 2500 units/mg. and the ACTH potency was less than 0·1 i.u./mg. By analysis of the material in the 24-tube apparatus [with equal volumes (8 ml.) of the above-mentioned solvents] the plot shown in Fig. 1 was obtained.

Comparison of the experimental curve with the theoretical calculated curve indicates a purity of 80–85 % for the sample of melanophore hormone examined. The material from the 'peak' tubes (nos. 6–10) of the 24-tube transfer after removal of the solvents was used for the amino acid determinations and the biological assay. The amino acid composition of the melanophore-expanding material from hog pituitary is indicated in Table 1.

jected to 100 transfers with equal volumes (100 ml.) of the above-mentioned solvents. Tubes 41–44 inclusive in this series contained the highest melanophore potency. The partition ratio was 0·74. The combined contents of the tubes, on evaporation of the solvents, yielded 18 mg. of dry residue which assayed 450 units/mg. An ACTH assay was not made on the material but, considering that a previous assay (Benfey, Saffran & Schally, 1954) on Stehle's preparation had indicated an ACTH potency of only 0·065 i.u./mg., it is safe to conclude that the activity of the fraction obtained after countercurrent purification probably was negligible. The plot of the data obtained from an analytical redistribution of the most potent material

Table 1. *Amino acid composition of the melanophore-expanding hormone from hog and ox pituitary*

| | Hog-pituitary extract | | | Ox-pituitary extract | | |
|---|---|---|---|---|---|---|
| Amino acid | % by wt. | Molar ratio | No. of residues/mol. | % by wt. | Molar ratio | No. of residues/mol. |
| Alanine | 0·2 | 0·2 | — | 2·6 | 2·9 | 3 |
| Arginine | 4·2 | 1·9 | 2 | 5·2 | 3·0 | 3 |
| Aspartic acid | 5·8 | 3·5 | 4 | 4·1 | 3·1 | 3 |
| Cystine | 0·3 | 0·1 | — | 1·6 | 0·7 | 1 |
| Glutamic acid | 7·3 | 4·0 | 4 | 7·5 | 5·1 | 5 |
| Glycine | 2·2 | 2·3 | 2 | 2·5 | 3·3 | 3 |
| Histidine | 2·9 | 1·5 | 2 | 4·2 | 2·7 | 3 |
| Isoleucine | — | — | — | 0·9 | 0·7 | — |
| Leucine | 0·3 | 0·2 | — | 3·7 | 2·8 | 3 |
| Lysine | 6·7 | 3·7 | 4 | 5·8 | 4·0 | 4 |
| Methionine | 1·6 | 0·9 | 1 | 0·8 | 0·5 | 1 |
| Phenylalanine | 3·7 | 1·8 | 2 | 5·3 | 3·2 | 3 |
| Proline | 4·9 | 3·4 | 3 | 3·8 | 3·3 | 3 |
| Serine | 2·2 | 1·7 | 2 | 2·3 | 2·2 | 2 |
| Threonine | — | — | — | 1·5 | 1·3 | 1 |
| Tryptophan | 5·3 | 2·1 | 2 | 3·7 | 1·8 | 2 |
| Tyrosine | 3·8 | 1·7 | 2 | 2·7 | 1·5 | 2 |
| Valine | 0·5 | 0·3 | — | 6·1 | 5·2 | 5 |

The tubes 166–168 inclusive in this series, on the other hand, were found to contain the highest ACTH activity. The combined contents of these tubes yielded 47 mg. of dry material which assayed only 20 units of melanophore-expanding potency/mg. and 14–15 i.u. of ACTH activity/mg. The plot of the data obtained from an analytical redistribution of the material between equal volumes (8 ml.) of 0·1 % trichloroacetic acid and *sec.*-butanol in the small Craig apparatus revealed that the material did not behave as a single substance. The partition ratio in this solvent system was approximately 1.

*Ox-pituitary extract*

A plot of the spectrophotometric data obtained with Stehle's ox-pituitary powder after countercurrent distribution between 0·1 % trichloroacetic acid and *sec.*-butanol revealed a single broad peak. In a larger run 317 mg. of the material was subwith equal volumes (8 ml.) of a 0·1 % trichloroacetic acid and *sec.*-butanol in the small Craig unit is shown in Fig. 2. The amino acid analysis of material from the 'peak' tubes (nos. 9–13) of the 24-tube transfer is indicated in Table 1. The partition ratios of the various preparations are given in Table 2.

It is of interest that treatment of the highly purified fraction from hog pituitary with alkali produced no increase in the activity whereas with the highly purified fraction from ox-pituitary alkali treatment produced approximately a threefold increase in the potency.

## DISCUSSION

Our ox and hog preparations are clearly different. One difference is the behaviour of the two preparations towards weak alkali. (As yet there is no explanation why the activity of certain melano-

phore-hormone preparations might be increased by treatment with alkali.) Secondly, the potency of the ox preparation (450 units/mg.) is much lower than that of the hog preparation (2500 units/mg.). This is approximately a fivefold difference in potency. The same ratio is seen in the starting acetone powder. Another striking difference is in the partition ratios between trichloroacetic acid and *sec.*-butanol. Further it seems that, from the amount of material which can be accounted for by the amino acid analysis (Table 1), the hog preparation contains more trichloroacetic acid than the ox preparation. (The preparations are in the form of trichloroacetic acid salts. Removal of the bound acid would accordingly result in increased potency/unit weight of material.) On the basis of the amino acid data the molar ratios have been calculated (Table 1). From these values it may be inferred that the minimum molecular weight of the hog preparation is approximately 3800 and that of the beef preparation 5600. Finally, the amino acid composition of our two preparations is different. Major differences between the two melanophore preparations are evident in the amounts of the amino acids alanine, leucine, threonine and valine. These differences between the two preparations may be due to different methods of production. It is possible that the method of purification influences the final product, since peptides are highly sensitive material. But it is also possible and probable that more than one peptide which exhibits melanophore potency may be isolated from pituitary tissue.

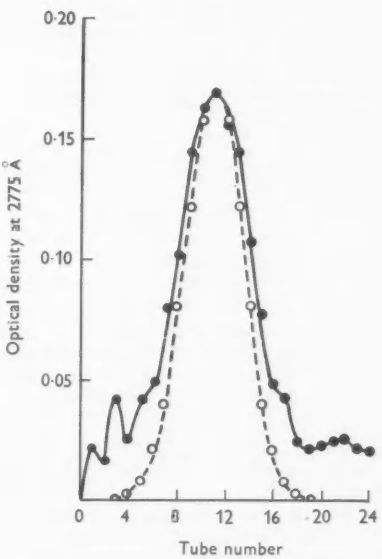


Fig. 2. Analytical redistribution of ox melanophore-expanding hormone between 0·1% trichloroacetic acid and *sec.*-butanol. ●, Experimental; ○, calculated.

In comparing our hog preparation with that of Lerner & Lee (1955), major differences in the amounts of the following amino acids are evident: alanine, cystine, histidine, leucine, methionine, threonine and valine. Although the two preparations appear to possess similar potencies, the differences in their amino acid composition lead the authors to conclude that the two preparations are not alike.

It has been claimed that the melanophore-expanding and the adrenocorticotropic hormone are identical, and supposedly pure ACTH preparations, including corticotropin B, have been reported to contain a high degree of melanophore potency (Brink *et al.* 1953; Winter, Brink & Folkers, 1953; Bell, 1954). Our countercurrent-distribution experiment with the hog material achieved a clear separation of the two biological activities. In addition, our ox-melanophore hormone contains only a trace of ACTH activity. This evidence leads us to conclude that the two hormones are not identical. [Raben *et al.* (1952) indicated that a separation of melanophore hormone and ACTH is possible by solvent partition, but this note has never been followed by any detailed description.] Apparently, in isolating corticotropin B, Kuehl *et al.* (1953), were not able to separate the two activities. As can be seen from Table 2, the partition ratio of our hog preparation is quite similar to that of corticotropin B (which is obtained from corticotropin by pepsin hydrolysis). Our ACTH preparation, on the other hand,

Table 2. *Partition ratios of melanophore-expanding and adrenocorticotropic hormone preparations between* 0·1 *and* 0·5% *trichloroacetic acid and* sec.*-butanol*

| | 0·1% trichloro-acetic acid | 0·5% trichloro-acetic acid |
|---|---|---|
| Hog melanophore-expanding hormone | — | 0·45–0·64 |
| Corticotropin B (Kuehl *et al.* 1953) | — | 0·5–0·6 |
| Hog adrenocorticotropic hormone | 1 | 7 |
| Corticotropin (Kuehl *et al.* 1953) | 1·31 | 7 |
| Ox melanophore-expanding hormone | 0·74–0·85 | 7 |

Table 3. *Amino acid composition of melanophore-expanding and adrenocorticotropic hormone preparations*

Molar ratios calculated for an arbitrary total of 50 amino acid residues per molecule.

| | Melanophore hormone | | | Corticotropic hormone | |
|---|---|---|---|---|---|
| Amino acids | Ox | Hog* | Hog† | Corticotropin B hog‡ | Corticotropin A hog§ |
| Alanine | 2·9 | 0·3 | 2·1 | 1·8 | 4·0 |
| Arginine | 3·0 | 3·2 | 3·0 | 5·2 | 4·0 |
| Aspartic acid | 3·1 | 6·0 | 3·8 | 3·3 | 3·0 |
| Cystine | 0·7 | 0·2 | 2·9 | — | — |
| Glutamic acid | 5·1 | 6·8 | 4·7 | 4·6 | 7·8 |
| Glycine | 3·3 | 3·9 | 4·1 | 4·8 | 4·0 |
| Histidine | 2·7 | 2·6 | 0·5 | 1·6 | 1·7 |
| Isoleucine | 0·7 | — | — | — | — |
| Leucine | 2·8 | 0·3 | 1·7 | — | 3·0 |
| Lysine | 4·0 | 6·3 | 4·7 | 6·4 | 5·3 |
| Methionine | 0·5 | 1·5 | — | 1·4 | 1·0 |
| Phenylalanine | 3·2 | 3·1 | 3·7 | 2·3 | 3·8 |
| Proline | 3·3 | 5·8 | 4·2 | 4·1 | 3·8 |
| Serine | 2·2 | 2·9 | 4·2 | 3·4 | 2·9 |
| Threonine | 1·3 | — | 1·4 | — | — |
| Tryptophan | 1·8 | 3·6 | 2·6 | 3·0 | 1·9 |
| Tyrosine | 1·5 | 2·9 | 3·0 | 3·1 | 1·9 |
| Valine | 5·2 | 0·5 | 3·3 | 4·7 | 3·2 |

* From Table 1, multiplied by 1·7.
† Percentage as given by Lerner & Lee (1955), multiplied by 131 and divided by the molecular weight of the amino acids.
‡ Data given by Brink *et al.* (1953), for the preparation isolated by countercurrent distribution, multiplied by 0·8.
§ Data given by Hays & White (1954) for the preparation 384–6–I, multiplied by 1·9.

behaves like unhydrolysed corticotropin. From this it may be inferred that the hog-melanophore hormone cannot be separated from corticotropin B in this solvent system but can be separated completely from corticotropin. This may account for the fact that corticotropin B possesses a high melanophore potency. (It has been known for many years that anterior pituitary lobes, which serve as source material for the isolation of ACTH, contain some melanophore activity.)

Most authors, including Lerner & Lee (1955) and Brink *et al.* (1953), report melanophore potency in terms of arbitrary units which are not related to Posterior Pituitary Standard Powder. This makes it difficult to compare the melanophore data of different authors.

The molar ratios of the component amino acids of various melanophore-hormone and ACTH preparations are listed in Table 3. For purposes of comparison they are recalculated to give an arbitrary total of 50 amino acid residues/molecule. From this it can be seen that our hog preparation differs from corticotropin B mainly by the absence of alanine and valine. Apart from these two amino acids the preparations are quite similar. In general, as can be seen from Table 3, melanophore and ACTH preparations show many common features in their amino acid composition.

## SUMMARY

1. The melanophore-expanding hormone has been isolated in highly purified form from ox and hog pituitary glands by countercurrent distribution. The materials are virtually free from adrenocorticotropic hormone, which has also been segregated.

2. An amino acid analysis and other evidence reveals numerous differences between the preparations from the two sources.

This study was supported in part by grants from the National Research Council of Canada to Professor K. I. Melville, from Canada Packers, Ltd., Toronto, to Professor O. F. Denstedt and one of the authors (J. L. P.), and from the Hutchison Fund of McGill University to the other author (B. G. B.). We are indebted to Professor R. L. Stehle for the donation of his preparation of pituitary and to Nordic Biochemicals, Ltd., Montreal, for the generous gift of acetone-dried hog posterior-pituitary powder. We are also grateful to Dr Gordon Grant and Mr J. Souch of Ayerst, McKenna and Harrison Ltd., Montreal, for making available the countercurrent instruments. We are indebted to Dr M. Saffran and Mr A. V. Schally, Allan Memorial Institute of Psychiatry, Montreal, for carrying out the ACTH assay.

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# Studies on the Skeletal Tissues

## 4. THE RENEWAL OF INORGANIC PHOSPHATE IN BONES OF VARIOUS SPECIES OF SMALL MAMMAL AS A FUNCTION OF TIME*

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(*Received 5 July* 1955)

The uptake of $^{32}P$ and $^{45}Ca$ by bones varies considerably with type, area and age of the tissue. Differences according to bone type (diaphysis, metaphysis, alveolus) are well established (Rogers & Weidmann, 1951); the faster rate of phosphorus renewal in *spongiosa* as compared with *compacta* was shown to be statistically significant in various species of small mammals (Rogers, Weidmann & Jones, 1953). Variations in the absorption of isotopes in the different areas of bone shaft were clearly demonstrated by radioautography (Amprino, 1952; Engfeldt, Engström & Zetterström, 1952) and also by measurements of radioactivity (Rogers *et al.* 1953). The age of bone greatly influences the rate of isotope incorporation. The uptake of minerals is much quicker in the developing bones of the young than in the mature bones of the adult animal (Leblond, Wilkinson, Bélanger & Robichon, 1950).

These variations were attributed to local structural differences in the bone tissue, such as differences in micro-structure (Amprino, 1952), crystal size (Hevesy, Levy & Rebbe, 1940), vascularity (Neuman & Riley, 1947), but also to differences in the chemical composition, e.g. degree of calcification (Hevesy *et al.* 1940; Amprino, 1952).

Isotope incorporation is comparatively rapid immediately after the administration of the tracer, and slows down markedly within a few hours. Hevesy *et al.* (1940) explained the initial phase as an ion-exchange reaction and the subsequent slower phase as a biological recrystallization. It was thought that the rate of reaction in the initial phase decreased with the exhaustion of the easily exchangeable fraction of bone-salt crystallites, and that the second phase was entirely governed by the rate of recrystallization. Neuman & Neuman (1953) defined recrystallization more explicitly as an ionic-diffusion through the solid crystal. One of the main reasons for regarding the initial uptake as an ion-exchange absorption was that the reaction was too fast to be accounted for by new bone formation and could, therefore, only indicate that an equilibrium was quickly established between the exchangeable ions of the surface area of the bone and the blood minerals. Bauer (1954), however, explained the decrease in turnover rates of bone salt by the rapid fall of isotope concentration in the blood. Since bone and blood minerals are in a dynamic equilibrium, bone deposits formed in the early phase of the reaction, when the tracer concentration of the blood is still high, contain more isotope than those formed in the later stages.

Radioautographic pictures showed heavy concentrations of $^{32}P$ and $^{45}Ca$ on the outside surfaces of the shaft, and fewer deposits in the Haversian systems. The areas of high tracer concentration coincided with areas of low mineral-salt content. It was suggested (Amprino, 1952; Engfeldt *et al.* 1952) that the turnover rate is proportional to the relative amount of recently formed but not yet fully calcified bone. The radioautographic findings were later confirmed by radioactivity measurements in bone-powder samples taken from the various regions of the shaft bone of the rabbit (Rogers *et al.* 1953). Significant differences were found in the turnover rates of phosphorus between subperiosteal, 'middle' and endosteal layers. These

* Part 3: Rogers, Weidmann & Jones (1953).

investigations are continued in the present work, in which a more efficient method for the extraction of newly labelled compounds from bone has allowed a more accurate measurement of the turn-over rates. The various layers of the shaft cortex of the rabbit and cat are compared, because the examination of their whole shaft bones showed a remarkable difference in the rate of tracer uptake (Rogers *et al.* 1953).

## METHODS

All samples of bone were taken from adult, healthy specimens of rabbits, cats and rats.

*Bone.* The preparation of bone samples has been reported previously (Weidmann & Rogers, 1950; Rogers *et al.* 1953). Carrier-free $H_3{}^{32}PO_4$ was administered intraperitoneally to the cat and the rat, but intravenously to the rabbit. Comparative measurements showed that, provided that the $^{32}P$ entered the circulation system, the route of administration made no difference to the specific activities of serum and bone phosphorus. In early rabbit experiments an almost complete loss of tracer occurred occasionally after intraperitoneal injection; consequently intravenous injection was adopted for all subsequent rabbit experiments. Measurements of radioactivity were carried out as described earlier (Rogers *et al.* 1953). The specific activity was expressed by the usual formula

$$\frac{(\text{Counts/min./mg. of P}) \times 10^8}{\text{Administered counts/min./kg. of body wt.}},$$

in which the theoretical count for $33\,\mu C = 7{\cdot}35 \times 10^7$ counts/min. was taken as administered count. The activity of the isotope solution was always measured before the injection into the animal.

### *Extraction of bone samples*

A method for the differential extraction of highly active bone-salt fractions from bone fragments (*a*) and bone-powder samples (*b*) has been devised, with saturated $(NH_4)_2SO_4$ solutions as solvents. For the differential extraction of bone fragments, tubular pieces of 1 in. length were selected from the central sections of femur and tibia. The technique of bone-powder sampling from the endosteal, subperiosteal and 'middle' layers of the shaft bone has been described in a previous paper (Rogers *et al.* 1953).

(*a*) *Differential extraction of bone fragments* (*paraffin-protection method*). All bone surfaces not to be extracted were covered with paraffin wax. For instance, when the outside layer was extracted the transverse section was covered with a thin layer of melted paraffin wax and the endosteal layer was protected by filling the marrow cavity entirely with wax; when the endosteal layer was examined, the outside surface and the transverse section were protected with a thin layer of wax. For the investigation of the 'middle' layer, shaft tubes of 1 in. length which were entirely covered with paraffin wax, were cut transversely into five smaller fractions to supply a sufficiently large transverse surface for the extraction. The middle layer includes, therefore, the entire cross-section of the shaft.

The bone fragments were shaken with 11 ml. of 90% saturated ammonium sulphate soln. for 1 hr. at room temperature, and samples of the solution were used for the estimations.

(*b*) *Differential extraction of bone powder.* Approximately 0·1 g. of bone powder was stirred vigorously with 3 ml. of saturated $(NH_4)_2SO_4$ soln. for 15 min. in the cold, and filtered through a small Büchner funnel. It was then washed twice with 3 ml. of saturated $(NH_4)_2SO_4$ soln. and three times with 3 ml. of distilled water. The solutions were combined and made up to 25 ml., and samples were taken for the phosphorus and radioactivity estimations. Phosphorus was estimated by a modification (Martland & Robison, 1926) of the Briggs method.

(*c*) *Repeated differential extraction of bone fragments.* In an attempt to determine the total uptake of $^{32}P$ in the two outside layers of cortical bone, the bone fragments were shaken four times as described above (*a*). As seen from Fig. 1, the specific activities decrease rapidly with each subsequent extraction, particularly in the short-term experiments. Hence, four successive extractions appear to be adequate to estimate all the labelled compounds in bone. By plotting the specific activities against the number of extractions, and extrapolating to an infinite number of extractions, an approximate value of the total $^{32}P$ content can be obtained through graphical estimation. The total $^{32}P$ uptake was used as an indicator of the amount of exchangeable phosphorus. Non-labelled phosphates, which were recently formed but not fully calcified, are dissolved simultaneously with the labelled compounds in saturated $(NH_4)_2SO_4$ solns. (Engfeldt *et al.* 1952). Phosphorus estimations showed that the amount of soluble phosphate decreases gradually in the four successive extractions. If the soluble phosphates represent the active fraction of bone minerals then the percentage exchangeable phosphorus can be calculated from the relationship:

$$\frac{\text{Total exchangeable P}}{\text{Extracted P}} = \frac{\text{Total } ^{32}P}{\text{Extracted } ^{32}P}.$$

Exchangeable P (g.) is expressed as a percentage of total P in the bone.

Table 1. *Comparison of specific activities in bones and bone parts of two groups of rabbits in different stages of development, measured 4 hr. after the injection of* $^{32}P$

Results represent mean values ± S.E.M. Figures in parentheses are the number of animals used. For definition of specific activities see Methods.

| Phase of development | Range of weights (g.) | Average sample of whole bone | | Differential extraction with paraffin wax-protection method from shaft bone (outer layer) |
|---|---|---|---|---|
| | | Cortical | Cancellous | |
| Mature | 1900–4200 | 46 ± 3 (29) | 324 ± 33 (21) | 565 ± 133 (9) |
| Growing | 2000–3400 | 99 ± 8 (8) | 821 ± 65 (8) | 1844 ± 267 (6) |

## RESULTS

### *Turnover rate of phosphorus in bones of different species of animal*

In a recent comparative study of fully grown rabbits, rats and cats, a relatively slow turnover rate of phosphorus was observed in the cortical bone of the cat (Rogers *et al.* 1953). It was stated then that the number of animals and the periods of observation were inadequate to define the species differences. In the present work more animals were used and the experimental period was extended to 30 days. The number of animals used to determine an experimental point varied between 3 and 44, and most results are based on at least six animals. Owing to the large variations in the mineral metabolism of bones between individual animals, representative mean values are difficult to establish. The specific activity of cortical and cancellous bones in some rabbits of normal adult weight was significantly above the average. In those cases the uptake of $^{32}P$ of the periosteal layer of the diaphysis was also rather high. The latter phenomenon is a characteristic of animals still in the process of growth (Weidmann & Rogers, unpublished work.) By comparing the figures obtained for immature with those for mature animals (Table 1) it will be seen that the probability that the two groups of animals belong to the same population is very small ($P < 0{\cdot}01$). Such animals were, therefore, not included in the assessment of means for normal adults.

Table 2 shows that the uptake of phosphorus by bones of the rabbit and rat is very fast, which is in good agreement with many previous findings, and that uptake of phosphorus is comparately slow in the cat. This is particularly evident during the first hours following the injection of the tracer. After 0·5 hr., about half of the ultimate total of tracer has been incorporated in the cortical bone of the rabbit and the rat, whereas the cat contains only about one-sixth of the maximum amount. As the tracer concentrations increase with time, the species differences diminish. It takes, however, several days in the cat to reach levels similar to those found in the rabbit and rat 4 hr. after the injection. The scatter of results is very large in long-term experiments; the high $^{32}P$ concentrations in the cortical bones of the rabbit and rat at the 30 days' interval are, therefore, difficult to appraise, and it is consequently uncertain whether the total uptake of $^{32}P$ in these species is in fact larger than in the cat.

The cancellous bones of all three species contain six to seven times more $^{32}P$ than the cortical bones; the rate of tracer incorporation is also faster. Species differences are similar to those found in the cortical bones. The initial reaction in cancellous

Table 2. *Specific activity of inorganic phosphorus in bones and serum of various animals, measured at different time intervals after the injection of* 33 $\mu$C/*kg. of body wt. of carrier-free* $^{32}P$ *as* $H_3{}^{32}PO_4$

Results represent mean values ± S.E.M. Figures in parentheses are the number of animals used. For definition of specific activity see Methods.

| | Cortical bone | | | Cancellous bone | | | Serum* | |
|---|---|---|---|---|---|---|---|---|
| Time | Rabbit | Cat | Rat | Rabbit | Cat | Rat | Rabbit | Cat | Rat |
| 10 min. | 6 ± 1 (3) | — | 19 ± 1 (5) | — | — | — | 134 200 | 127 000 | 100 000 |
| 0·5 hr. | 34 ± 4 (11) | 7 ± 1 (6) | 28 ± 3 (6) | 306 ± 61 (7) | 64 ± 6 (5) | — | 149 000 | 151 000 | 82 000 |
| 4 hr. | 46 ± 3 (29) | 29 ± 4 (18) | 65 ± 3 (44) | 324 ± 34 (21) | 286 ± 47 (15) | 373 ± 18 (42) | 21 900 | 33 600 | 10 600 |
| 1 day | 45 ± 12 (4) | — | 43 ± 4 (3) | 315 ± 100 (3) | — | — | 7 840 | 12 600 | 2 300 |
| 9 days | 61 ± 10 (13) | 50 ± 12 (7) | 45 ± 4 (5) | 534 ± 113 (8) | 466 ± 91 (5) | — | 1 980 | 2 800 | 1 100 |
| 30 days | 85 ± 12 (10) | 50 ± 14 (8) | 94 ± 14 (8) | 469 ± 140 (11) | 488 ± 105 (7) | 204 ± 47 (7) | 982 | 800 | 2 550 |

* Variation of S.E.M. ≃ 11 %.

bones is again comparatively slow in the cat, proving that the slow rate of mineral-salt renewal is a general characteristic of cat bones.

The specific activity of the cortical bones, after reaching a maximum, does not decrease in the course of 30 days in any of the three species investigated. No losses of isotope were observed either in the cancellous bones of the rabbit or of the cat. The only decrease of isotope concentration occurred in the trabeculae of the rat, which lost almost half their original uptake. This is most likely due to an extensive resorption of these bones, whose mineral salt is utilized for skeletal growth. In contrast to the cat and the rabbit skeletal growth does not cease in the adult rat (Singer, Armstrong & Premer, 1952). The present results confirm, therefore, the irreversible incorporation of $^{32}P$, and this is true for the cancellous bones, which are most active biologically, as well as for the cortical bones. According to Neuman & Mulryan (1952) the apparent lack of equilibrium between bone and plasma minerals is due to a recrystallization of bone-salt crystallites, whereby the isotope is rendered unavailable for exchange reactions.

The specific activity of serum decreases rapidly in all three species, the rate being greatest in the rat and least in the cat, whereas the rabbit occupies a middle position. There appears to be a direct connexion between the decrease of tracer concentration in the serum and the isotope incorporation into the bones, particularly so in the period between 0 and 4 hr. In the cat, where the tracer disappears most slowly from the serum, the uptake of $^{32}P$ by the bones is also slowest, whereas both processes are quickest in the rat. A comparatively high specific activity was observed in the serum of the rat at the 30 days' interval which is, presumably, due to the partial dissolution of the labelled trabeculae discussed above.

### *Uptake of phosphorus into different parts of long bones*

The problem whether inorganic phosphorus of serum or other compounds, such as organic phosphate esters, react directly with bone phosphorus, is of considerable interest. Intermediate reactants should be recognizable by their comparatively high specific activities. In an attempt to obtain information about possible percursors, samples of bone powder were extracted with chemically inert salt solutions. Various salt solutions were tested, and sodium acetate buffers (pH 5–6) gave the best results. Since the completion of these experiments, Zetterström (1952), with saturated ammonium sulphate solns. as solvents, succeeded in extracting fairly active fractions from bones of rabbits and rats. Ammonium sulphate is a very good separating agent, probably because of its acid reaction (pH of saturated ammonium sulphate is 3·8), and the capacity of the sulphate ion to form a comparatively insoluble calcium salt, thereby replacing the phosphate. External conditions, such as time and intensity of agitation, or temperature of the solution, have, within reasonable limits, very little effect on the extraction. A modified version of the technique of Zetterström (1952) was applied in these experiments.

*Differential extraction of bone fragments.* In preliminary experiments the cortex of bone shaft was subdivided into outer, 'middle' and inner layers by the usual procedure (Rogers *et al.* 1953) and the bone-powder samples were subjected to differential extraction. The arbitrary separation of the shaft into three layers by means of a dental burr can never be wholly satisfactory, however thin the burred-off layer may be; some fraction of the less-active middle area will always be contained in the endosteal and periosteal samples. By using bone fragments for the differential extraction with the paraffin wax-protection method highly active fractions were obtained, particularly from the endosteal area, the specific activity of which was occasionally 200 times that of the bone average. The differential extraction by the paraffin wax-protection method seems to select the newly labelled compounds very efficiently.

Table 3 contains the specific activities of rabbit bones and bone parts. The three areas of the shaft

Table 3. *Comparison of the specific activities of inorganic phosphorus in bones of the rabbit obtained by various methods*

Results represent the mean values ± S.E.M. Figures in parentheses are the number of animals used. The figures for middle and inner layers are the same as for the outer layer. For definition of specific activities see Methods.

| Time | TCA-extract of shaft (average) (1) | TCA-extract of bone powder from various layers of the shaft: Outer (2) | Middle (3) | Inner (4) | Differential extract of the various layers of bone shaft: Outer (5) | Middle (6) | Inner (7) | TCA-extract of metaphysis (average) (8) | Differential extract of bone powder from metaphysis (9) |
|---|---|---|---|---|---|---|---|---|---|
| 0·5 hr. | 34 | 41±5 (7) | 33±5 | 182±42 | 854±178 (4) | 141±23 | 5050±1030 | 306 | 2663±689 (7) |
| 4 hr. | 46 | 43±4 (8) | 24±3 | 141±53 | 654±133 (9) | 149±30 | 3910±666 | 324 | 2100±376 (7) |
| 1 day | 45 | 51±7 (3) | 26±2 | 117±18 | 504±165 (3) | 63±7 | 1254±281 | 315 | 855±239 (3) |
| 9 days | 61 | 66±17 (6) | 29±4 | 116±14 | 242±46 (3) | 106±21 | 803±60 | 534 | 693±104 (8) |
| 30 days | 85 | 103±10 (10) | 38±4 | 123±17 | 972±111 (5) | 148±28 | 892±127 | 469 | 610±40 (11) |

were examined by differential extraction of bone fragments, as well as by dissolving bone powder samples in 20% trichloroacetic acid (TCA). By presenting these two sets of results side by side the appraisal of turnover reactions is facilitated. A comparison of the specific activities in the differential extracts of the three layers with that of the shaft average (first column) shows that the latter conveys a wholly inadequate picture of the complexity of turnover reactions. The process of labelling varies distinctly with the area of bone, especially in the first hours after the injection; e.g. the ratio of specific activities at the 0·5 hr. interval varies between 6:1:36 for the outer:middle:inner layers (Table 3, columns 5–7). The endosteal area has a very fast rate of tracer uptake which was first demonstrated by the present technique. In the rabbit endosteum maximum uptake is reached almost immediately after the administration of the total $^{32}P$ concentration. Hence, the fall in the specific activities of P in ammonium sulphate extracts is also due to decreased solubility of the newly labelled phosphates. The specific activities of the cancellous bones are relatively lower than those of the endosteal area of the shaft, which, considering the high biological activity of the cancellous bones, is rather surprising. The two sets of results are, however, not strictly comparable because the former were obtained by differential extraction of bone-powder samples and the latter from bone fragments. In powders there is an appreciable co-dissolution of non-labelled phosphates, which naturally reduces the specific activity. This does not occur to the same extent in bone fragments, where a solid surface is exposed to the solvent action.

The results of the examination of cat bones are contained in Table 4. A comparison with Table 3

Table 4. *Comparison of the specific activities of inorganic phosphorus in bones of the cat obtained by various methods*

Results represent mean values ± S.E.M. Figures in parentheses are the numbers of animals used. The figures for middle and inner layers are the same as for the outer layer. For definition of specific activities see Methods.

| Time | TCA-extract of shaft (average) | TCA-extract of bone powder from various layers of the shaft | | | Differential extract of the various layers of bone shaft | | | TCA-extract of metaphysis (average) | Differential extract of bone powder from metaphysis |
|---|---|---|---|---|---|---|---|---|---|
| | | Outer | Middle | Inner | Outer | Middle | Inner | | |
| 0·5 hr. | 7 | 8±1 (5) | 8±2 | 18±3 | 179±38 (5) | 32±5 | 778±185 | 72 | 550±96 (7) |
| 4 hr. | 24 | 25±6 (7) | 16±5 | 48±8 | 477±70 (6) | 80±14 | 2180±313 | 284 | 2160±310 (10) |
| 9 days | 50 | 54±18 (3) | 32±10 | 72±20 | — | — | — | 466 | 803±190 (3) |
| 30 days | 50 | 81±23 (7) | 43±8 | 104±35 | 186±8 (3) | 67±13 | 268±81 | 488 | 581±100 (5) |

tracer, followed by a sharp decrease of activity. It could be assumed that this decrease in activity is exclusively due to rapid bone resorption in the endosteal layer. This possibility is ruled out, however, by the fact that at the 30 days' interval the percentage decrease of specific activity in the differential extract is 82% (Table 3, column 7) as compared with the loss of only 32% (Table 3, column 4) in the total tracer concentration. The decrease in the specific activity in the differential extract is therefore mainly due to the reduced solubility of labelled phosphates.

Predominance of the endosteal activity is a characteristic feature in the process of bone renewal of the adult animal. In single-injection experiments the amount of tracer uptake is much bigger in the endosteum than in the periosteum. Another important difference between the two layers is that the tracer concentration falls in the inner area with time, whilst it increases in the outer layer.

The tracer-uptake in the metaphyseal trabeculae of the rabbit is as fast as in the endosteum of the diaphysis. The isotope concentration decreases sharply in the differential extracts after the 0·5 hr. interval, but no concomitant losses occur in the shows that, generally, much more active fractions can be extracted from the rabbit than from the cat. The comparatively slow onset of bone-salt renewal in the cat is very clearly demonstrated with the paraffin wax-protection method. At the 0·5 hr. interval all three layers of the bone shaft and the cancellous bones of the cat contain only a fraction of the tracer amount present in the rabbit at the same time interval. Another interesting difference between the two species is the increase of the phosphorus uptake in the cat between 0·5 and 4 hr., as opposed to the decrease in the rabbit. A comparison of the TCA-extracts from bone-powder samples of the three layers of the two species also shows that in the cat the process of labelling progresses at a much slower rate than in the rabbit. The changes in specific activity can be explained in the same way as in the rabbit.

### *Influence of time upon the solubility of newly labelled compounds*

The decrease of solubility of the labelled phosphates was explained by Amprino (1952) and Zetterström (1952) as a progressive mineralization of fresh bone-salt deposits. This process can be

conveniently measured by comparing specific activities in the differential extracts of bone-powder samples with those of the TCA-extracts of the residue.

*Cancellous bone.* Table 5 shows that the tracer distribution becomes rapidly uniform throughout the trabecular spicules of the rabbit. In the early phases of the reaction there are large differences between the specific activities of the easily soluble, reaction affects the entire mass of trabecular spicules, and that these bones are renewed within the remarkably short time of 9 days.

The processes are largely the same in the cat (Table 5), but the rate of transformation of new mineral deposits into less-soluble compounds is markedly slower, uniformity being reached only after 30 days. This is further evidence for the comparatively slow bone-salt renewal in the cat.

Table 5. *Influence of time upon the solubility of labelled phosphorus compounds in the trabecular spicules of the metaphyses of rabbits and cats*

Results are expressed as specific activity and represent mean values ± S.E.M. For definition of specific activity see Methods. Figures in parentheses are the number of animals used. The figures for differential extraction are the same as for whole bone.

| | Rabbit | | | Cat | | |
|---|---|---|---|---|---|---|
| | | Differential extraction of bone-powder samples | | | Differential extraction of bone-powder samples | |
| Time | TCA-extract of whole bone (average) | $(NH_4)_2SO_4$ fraction | TCA-extract of residue | TCA-extract of whole bone (average) | $(NH_4)_2SO_4$ fraction | TCA-extract of residue |
| 0·5 hr. | 306 (7) | 2663 ± 689 | 135 ± 16 | 64 (5) | 526 ± 96 | 56 ± 6 |
| 4 hr. | 342 (9) | 1104 ± 331 | 114 ± 15 | 226 (7) | 2042 ± 317 | 197 ± 69 |
| 1 day | 315 (3) | 855 ± 239 | 257 ± 89 | — | — | — |
| 9 days | 534 (8) | 693 ± 104 | 607 ± 146 | 477 (3) | 803 ± 190 | 470 ± 116 |
| 30 days | 477 (11) | 601 ± 40 | 501 ± 51 | 512 (5) | 581 ± 100 | 465 ± 105 |

Table 6. *Influence of time upon the solubility of labelled phosphorus compounds in the various layers of cortical bone of adult rabbits*

Results are expressed as specific activity and represent mean values ± S.E.M. Figures in parentheses are the number of animals used. The figures for differential extraction are the same as for whole bone.

| | Outer layer | | | Inner layer | | |
|---|---|---|---|---|---|---|
| | | Differential extraction of bone-powder samples | | | Differential extraction of bone-powder samples | |
| Time | TCA-extract of whole layer (average) | $(NH_4)_2SO_4$ fraction | TCA-extract of residue | TCA-extract of whole layer (average) | $(NH_4)_2SO_4$ fraction | TCA-extract of residue |
| 0·5 hr. | 41 (5) | 159 ± 27 | 24 ± 3 | 152 | 678 ± 197 | 61 ± 7 |
| 4 hr. | 54 (7) | 172 ± 17 | 29 ± 5 | 160 | 572 ± 209 | 77 ± 18 |
| 1 day | 51 (3) | 248 ± 31 | 44 ± 7 | 117 | 381 ± 64 | 117 ± 32 |
| 9 days | 122 (8) | 278 ± 73 | 110 ± 38 | 160 | 327 ± 17 | 103 ± 21 |
| 30 days | 163 (8) | 285 ± 48 | 174 ± 51 | 164 | 301 ± 22 | 180 ± 23 |

recently labelled compounds in the differential extracts and the old-established bone-salts in the TCA-solute of the residue, but these differences have already decreased 24 hr. after the injection. The fall in the specific activities in the differential extracts is not due to an overall loss of radioactivity, as shown by the almost constant radioactivity of the TCA-extracts of whole bones, but is caused by a transformation of recent deposits into less-soluble compounds, as seen from the increasing specific activities in the residues. In the rabbit the differences are completely absent 9 days after the injection of the isotope, as indicated by the similar specific activities of total bone solute, ammonium sulphate fraction and residue. This implies that the

*Cortical bone.* Bone-powder samples of the two outside layers of rabbit shaft were examined by differential extraction, and a somewhat different picture from the cancellous bones was obtained (Table 6). The specific activity in the ammonium sulphate extracts of the endosteum decreases during the course of 30 days, but it always remains distinctly above that of the residue, which means that the process of renewal is not completed within 30 days in the endosteum. The explanation for the changes in solubility may be the same as those given for similar changes in the cancellous bones.

In contrast to the endosteum, the total isotope concentration increases in the periosteum. Since the specific activities of both fractions, ammonium

sulphate and residue, and the total $^{32}P$ content increase simultaneously, it is quite obvious that, apart from a gradually decreasing solubility of the first deposits, an additional incorporation of $^{32}P$ takes place in the periosteum in the later phases. Thus additional evidence was obtained for the interesting difference in the biological activity between the two surface areas of the shaft.

*Total $^{32}P$-uptake by the two outside layers of bone shaft and its relationship to the percentage of exchangeable phosphorus*

As seen from Fig. 1, the incorporated $^{32}P$ can be extracted almost completely by repeated differential extractions, particularly if the deposits are of recent origin. From 0·5- and 4-hr.-old deposits the first elution removes approximately 75 % of the incorporated $^{32}P$. The extraction is less effective as the labelled phosphates in bone become older; from 30-day-old deposits the first elution removes only about 30 % of the total $^{32}P$ present in bone. When the $^{32}P$-uptake is used as an indicator, it is possible to calculate the percentage of exchangeable phosphorus. The calculation is based on the fact that non-labelled phosphates are dissolved together with $^{32}P$-compounds, and that these soluble phosphates are regarded as corresponding to the exchangeable part of bone salts (see Methods). The values for exchangeable phosphorus contained in Table 7 represent only part of the total active phosphorus in bone shaft because the 'middle' area is not included. Since the bulk of $^{32}P$ is concentrated in the two outside layers, the sum of exchangeable phosphorus in the two outside layers (columns 3 and 6) forms the major fraction of the active bone salts.

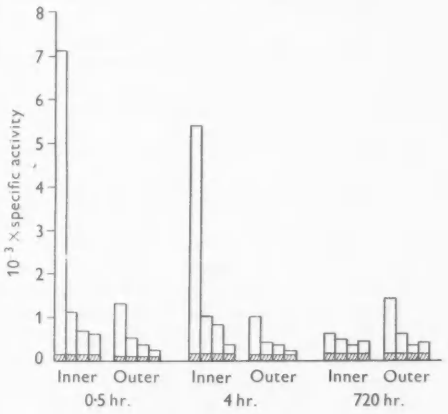


Fig. 1. Repeated differential extractions from the inner and outer layers of the cortical bone of the rabbit at different time intervals after the injection of radioactive phosphate. Shaded areas represent the average specific activity of the layer.

The percentage of exchangeable phosphorus varies between 3·29 and 4·73 in the rabbit, and from 2·24 to 4·41 in the cat when measured at different time intervals after the administration of the tracer. Since the active fraction of bone salt is a constant value, the true value lies, presumably, between the two figures. An assessment of the active phosphates is rendered difficult owing to the fact that the ammonium sulphate extract contains bone salts of different solubilities (Zetterström, 1952), and consequently of differing exchangeabilities. The $^{32}P$ reacts in the first place with the most reactive fraction of bone minerals, and thus, by calculating the exchangeable phosphorus in relation to the $^{32}P$, present at the 0·5 hr. interval, the less-active fractions are not taken into consideration, i.e. the 0·5 hr. results are probably too low. On the other hand, the values calculated from the 30-day-old $^{32}P$ deposits are too large, because part of the labelled phosphates have already become insoluble and can no longer be accounted for as easily exchangeable phosphorus. In view of these limitations of the method, the results are in satisfactory agreement with the figure (5 %) quoted by Neuman & Weikel (1954) for the exchangeable pool.

The active fraction is higher in the endosteum than in the periosteum in both species. On both surfaces the rabbit shows a higher percentage of exchangeable phosphorus than the cat.

Table 7. *Percentage of exchangeable phosphorus in the two outside layers of cortical bone of the rabbit and cat at different time intervals after the injection of radioactive phosphate*

Results are expressed as percentage of total phosphorus in shaft bone.

| | Rabbit | | | Cat | | |
|---|---|---|---|---|---|---|
| Time | Subperiosteal layer | Endosteal layer | Sum in both layers | Subperiosteal layer | Endosteal layer | Sum in both layers |
| 0·5 hr. | 1·58 | 1·71 | 3·29 | 0·92 | 1·32 | 2·24 |
| 4 hr. | 2·02 | 2·27 | 4·29 | 0·68 | 0·66 | 1·34 |
| 30 days | 2·15 | 2·58 | 4·73 | 2·02 | 2·09 | 4·11 |

## DISCUSSION

In mature animals, where no growth occurs, ionic-exchange, re-crystallization and Haversian replacement were suggested as the only mechanisms regulating the mineral-salt uptake (Neuman & Weikel, 1954). A rapid initial tracer uptake occurs in the diaphyseal and metaphyseal parts of the bone shaft, which confirms previous findings. This is, however, not a general phenomenon because the initial uptake in the cat is relatively slow. The main distinguishing feature between the cat and the two other species is not in the extent, but in the rate, of renewal. Hence, the differences cannot be explained by variations in the amount of exchangeable bone-salt crystallites. The slow incorporation of $^{32}P$ into the bones of the cat in the first phase of the reaction is not due to lack of available phosphate, because the specific activity of the serum inorganic phosphate is not lower than that of the two other species. Turnover rates of phosphorus were also compared in the bone marrow of the cat and the rabbit. The acid-soluble phosphate compounds of bone marrow are more slowly renewed in the cat, but the differences between the two species are too small to account for the large differences in bone metabolism (unpublished results). A slow phosphorus turnover is, however, not a general characteristic of the cat; the turnover rates of the phosphorus compounds in the liver, for instance, are above those of the rabbit (unpublished results). It appears, therefore, that a local factor at the site of ossification is responsible for the species differences in bone-salt renewal.

By applying an improved technique for the separation of labelled phosphates from old-established bone, much new information was obtained about the mode of bone-salt renewal in the adult animal. Turnover reactions are far better demonstrated when, instead of the TCA-extracts from average samples of whole bone, differential extracts of bone parts are used for the measurement of radioactivity. The species differences are biggest in the areas most active biologically: the endosteal surface of bone shaft and the trabeculae of the metaphysis. In the past, quantitative differences in the tracer incorporation between cancellous and cortical bones were explained, with some justification, by structural variations between the two types of bone. The same reasoning is, however, hardly applicable to the differences found in the corresponding bones of the two species. It is particularly difficult to see how surface factors could cause such large differences in the rate of renewal, as observed in corresponding bone areas of the cat and the rabbit.

Apart from the much bigger initial $^{32}P$-uptake, the progress of labelling differs distinctly in the rabbit and cat. In the cat the quickest uptake occurs several hours after the injection of the tracer, and is then four to five times larger than in the first 0·5 hr. This is not compatible with the view that ionic-exchange absorption in the easily exchangeable fraction accounts for the fast initial phase of the reaction. The delayed uptake in the cat could be due to a comparatively slow diffusion across membrane barriers, or to a low turnover rate of an organic-phosphate precursor, but there is no evidence for this.

When the rate of the initial reaction is high, the solubility of the labelled compounds also decreases more rapidly. Considering that subsequent $^{31}P$-deposits render the labelled compounds more insoluble, there is no need to assume different reaction mechanisms for initial uptake and re-crystallization. The high turnover rate of the endosteal area of the shaft and the cancellous bones is due, not to a quicker re-crystallization, but to a more efficient biological functioning which regulates both the initial uptake and the decrease in solubility by the same mechanism. Accretion of bone salt (Haversian replacement) is in principle not different from these reactions; where the initial reaction is quicker more bone salt will be deposited eventually.

The increasing insolubility of labelled phosphates is best demonstrated by repeated differential extractions, carried out at different time intervals after the administration of the tracer. Two reasons could account for the change in solubility: (*a*) the isotopes migrate into the deeper strata of crystals; (*b*) a progressive mineralization of the newly deposited bone salt takes place, whereby the highly labelled compounds become buried under layers of non-labelled phosphates. The fact that the tracer distribution rapidly reaches uniformity in the cancellous bones favours the second explanation. It is not likely that the speed of any type of ion-migration is such as to advance throughout the whole thickness of a trabecular spicule within a few days, as was found in the rabbit. The rapid total renewal of the cancellous bones is, presumably, partly due to the specific lamellar arrangement in the microstructure (Amprino & Engström, 1952) which affords an easy access of the body fluids to the bone-salt crystallites within the bone. The surfaces of the crystal units are quickly labelled, followed by equally quick $^{31}P$ deposition, which decreases the solubility of the newly labelled compounds uniformly throughout the spicule. Owing to the dynamic equilibrium between bone and blood minerals, equal amounts of bone salt are dissolved as deposited, but losses of $^{32}P$ are not observed because of the large $^{31}P/^{32}P$ ratio. Favourable structural arrangements may explain the quicker rate of renewal and the deeper range of

reaction in cancellous as compared with cortical bones, where the reaction is restricted to a few surface layers, but it does not explain the difference in the rate of renewal between the cancellous bones of the rabbit and cat. There must be other factors, apart from structural ones, on which the rate of mineral salt turnover depends.

Bone-salt renewal occurs in mature shaft bones mainly on the marrow side, i.e. in the same area where resorption is known to take place. Whether the two processes are connected with each other, e.g. through strain and stress, cannot be decided from the present experiments. But the close vicinity of such opposite processes rather suggests a cellular regulation (Newton, 1939).

## SUMMARY

1. The rate of tracer uptake in long bones varies according to species; the incorporation of $^{32}P$ is considerably slower in the cat than in the rabbit or rat.

2. No decrease of tracer concentration occurs in the cortical parts of the long bones of the rabbit, cat or rat, or in the cancellous parts of the two former species in the course of 30 days. The decrease of the specific activity of phosphorus in the cancellous bones of the rat is explained by the continued skeletal growth of this animal in adult age.

3. The species differences are much better demonstrated with a newly developed technique, which affords an almost complete separation of the newly labelled bone salts from old-established bone. Large differences were found in the function of various parts of the shaft.

4. The different aspects of bone renewal in the adult animal, such as initial uptake, recrystallization, Haversian replacement, mobilization of fixed isotopes, equilibrium between bone and blood minerals, are discussed in the light of the new observations.

I take great pleasure in thanking Dr H. J. Rogers for his continued interest in this work and for many helpful suggestions. Thanks are also due to Mr R. W. Lofthouse and Miss E. B. Morley for excellent technical assistance.

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# Determination of Cocarboxylase in Blood

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(*Received* 25 *July* 1955)

Cocarboxylase estimation has received considerable attention from Ochoa & Peters (1938) and Westenbrink & Steyne-Parvé (1949), among others. Most of the accepted methods employ alkali-washed yeast apoenzymes activated by excess of thiamine, and some also include iodoacetate to prevent the phosphorylation of added thiamine (Blanchaer & Cohen, 1949). Goodhart & Sinclair (1939) developed a method for estimating cocarboxylase in heated blood, and Westenbrink & Steyne-Parvé (1949) used acid to extract cocarboxylase from blood, before applying their 'two-stage' method for assay.

In a system such as heated blood, which contains diphosphopyridine nucleotide and adenosine triphosphate in sufficient quantity to allow cocarboxylase synthesis by yeast enzymes (Lipton & Elvehjem, 1939), it seems unwise to include excess of thiamine. Further, now that carboxylase is recognized as a sulphydryl enzyme, which is

inhibited by iodoacetate (Stoppani, Actis, Deferrari & Gonzalez, 1953), it seems reasonable to exclude iodoacetate also from cocarboxylase-assay systems.

We have found alkali-washed yeasts to be unreliable sources of apoenzymes; with substances contained in blood they gave side reactions that could result in spurious evolution of $CO_2$. It was therefore decided to apply a purified apocarboxylase preparation (Green, Herbert & Subrahmanyan, 1941) in the study of cocarboxylase estimation, in the hope that a better assay system could be produced.

The method to be described here introduces the use of Norite charcoal to eliminate an enzyme blank by adsorbing the cocarboxylase from alkaline solution during enzyme preparation, and the use of cyanide or thiosulphate to overcome the effects of interfering substances, probably trace metals. An internal standard ensures identical conditions of activation in tests and standards, as both contain heated blood.

## METHOD

### *Principle*

Cocarboxylase in heated blood cells is recombined with apocarboxylase prepared from yeast, and the resulting holoenzyme is employed in the decarboxylation of pyruvic acid, by a manometric technique for the measurement of evolved $CO_2$.

### *Reagents*

*Stock cocarboxylase standard*, 100 $\mu$g./ml. This can be kept at 4° for up to 4 weeks.

*Dilute standard cocarboxylase.* This is freshly prepared from stock so as to contain 0·1 $\mu$g./ml. of phosphate buffer (pH 6·2).

0·1 M *Phosphate buffer*, pH 6·2. This is made by mixing 203·4 ml. of 0·1 M-$KH_2PO_4$ solution with 46·6 ml. of 0·1 M-$Na_2HPO_4$ solution.

0·33 M *Sodium pyruvate.* 2·0 ml. of pyruvic acid (purest commercial) is neutralized in water to pH 6·2 by adding about 2·5 ml. of 50% (w/v) NaOH and the whole is then diluted to 100 ml. with phosphate buffer, pH 6·2.

0·2 M *Sodium cyanide.* 1% (w/v) NaCN in phosphate buffer is adjusted to pH 6·2 with $H_3PO_4$.

*Ionic-activating solution.* 1 vol. of 0·1 M-$MnSO_4$ solution is mixed with 9 vol. of 0·1 M-$MgCl_2$ solution.

M *Calcium acetate solution.*

*Apocarboxylase solution.* This is prepared at the time of the experiments. Stock enzyme suspension (10 ml., prepared as described below) is centrifuged for about 0·5 hr. To the precipitate are added 1 ml. of ionic-activating solution, 1 ml. of 0·2 M-NaCN solution and 8·5 ml. of phosphate buffer, pH 6·2. A solution of activated apocarboxylase, sufficient for ten Warburg vessels, is then obtained.

*Stock enzyme.* (*a*) *Stock yeast.* About 0·5 lb. of ordinary baker's yeast is washed with 5 vol. of tap water, filtered on a large Büchner funnel (Whatman no. 541 or 542 paper), broken up and dried thoroughly in a vacuum desiccator over conc. $H_2SO_4$. This yeast keeps almost indefinitely. Brownish, hard yeasts usually give the best enzyme preparations.

(*b*) *Suspension.* Dried yeast (20 g.), finely powdered, is stirred into 180 ml. of 0·1 M-$Na_2HPO_4$ (pH 8·5) at 37°. The smooth suspension formed is incubated at 37° for 2 hr. so that the carboxylase is released into the solution.

The yeast cells are centrifuged and washed once with twice their volume of phosphate solution (pH 8·5) before being finally rejected. The combined supernatants are adjusted to pH 9·0 with conc. $NH_3$ soln. (sp.gr. 0·880) and 4 ml. of M calcium acetate are stirred in to remove turbidity (Green *et al.* 1941). The calcium phosphate precipitate is removed by centrifuging for 2 min. To the cleared solution solid $(NH_4)_2SO_4$ (38 g./100 ml. of solution) is added to precipitate the enzyme, which is separated in the centrifuge at high speed for 1 hr.

The precipitated protein is then dissolved in 50 ml. of 0·1 M-$Na_2HPO_4$ solution and, after adjustment to pH 9·0 with ammonia, 700 mg. of Norite charcoal is added and the mixture is stirred for about 10 min. to remove cocarboxylase dissociated from the enzyme. The charcoal is removed by centrifuging for 2 min., and the solution is treated with 2·0 ml. of M calcium acetate, as before, to clear it. Any floating material is removed by filtering the solution through a rapid paper, and $(NH_4)_2SO_4$ (38 g./100 ml. of solution) is again used to precipitate the enzyme. The resultant suspension, which should be adjusted to pH 6·2 with dilute HCl, is almost white and gives an enzyme of negligible blank. This suspension keeps for about 4 weeks at 4°. If the precipitated enzyme is separated, dissolved in phosphate buffer (pH 6·2) and freeze-dried, it keeps almost indefinitely (Murfitt, 1954).

### *Treatment of blood*

Well-mixed heparinized blood (4 ml.) is transferred to a tube and centrifuged. After the volume has been marked, the plasma is rejected and accurately replaced by phosphate buffer, pH 6·2. The cells are resuspended in the solution by stirring with a glass rod, and, after the tube is stoppered firmly, it is heated for 3 min. in a boiling-water bath. When cool, 4 ml. of buffer is added and a smooth suspension obtained by stirring with a glass rod. Since plasma contains no cocarboxylase, 1 ml. of heated blood suspension is equivalent to 0·5 ml. of original whole blood.

### *Procedure*

The assay procedure is conducted in duplicate in Warburg vessels to which the reactants are added in order, as indicated in Table 1.

For apoenzymes prepared with Norite charcoal the blank flasks are generally unnecessary.

After the Warburg manometers have been assembled they are equilibrated in the bath at 30° for 15 min. to allow time for holoenzyme formation. Afterwards pyruvate is tipped in from the side bulb at zero time, the taps are closed at 5 min., and readings are taken at 10 and 12 min. and then again at 40 and 42 min. This gives readings during steady evolution of $CO_2$ for two overlapping 30 min. periods, from which mean values can be obtained.

### *Calculation*

Evolution is measured in $\mu$l. of $CO_2$/30 min.

If $T$ represents the test evolution, $B$ the blank evolution and $S$ the internal-standard evolution of $CO_2$,

$$\text{Blood cocarboxylase} = \frac{T-B}{S-T} \times 10\,\mu\text{g./100 ml.}$$

Table 1. *Blood-cocarboxylase assay system*

Warburg flasks are set up in duplicate with the materials listed, and $CO_2$ evolution at 30° is measured for 30 min. after addition of the pyruvate to the enzyme–blood–buffer mixture.

| | Blank (ml.) | Test (ml.) | Internal standard (ml.) |
|---|---|---|---|
| Dilute cocarboxylase (0·05 μg.) | — | — | 0·5 |
| Phosphate buffer, pH 6·2 | 1·5 | 0·5 | — |
| 0·33 M Pyruvate (side bulb) | 0·5 | 0·5 | 0·5 |
| Heated blood suspension | — | 1·0 | 1·0 |
| Activated enzyme solution | 1·0 | 1·0 | 1·0 |

Table 2. *Effect of various additions upon cocarboxylase activity*

The enzyme control flask contained apocarboxylase and pyruvate. Other flasks contained 1 μg. of cocarboxylase in addition. The final volume in all flasks was made up to 3 ml. with phosphate buffer.

| System | Additions (0·1 ml. of 0·1 M) | Evolution of $CO_2$ (μl./30 min.) |
|---|---|---|
| Enzyme control | — | 24 |
| Control + cocarboxylase | — | 24 |
| Control + cocarboxylase | Hexose diphosphate | 25 |
| Control + cocarboxylase | Glycine | 28 |
| Control + cocarboxylase | Citrate | 37 |
| Control + cocarboxylase | NaCN | 41 |

## RESULTS

Over the usual range of working conditions and up to 0·2 μg. of cocarboxylase, $CO_2$ evolution bears a linear relationship to quantity of cocarboxylase (Fig. 1).

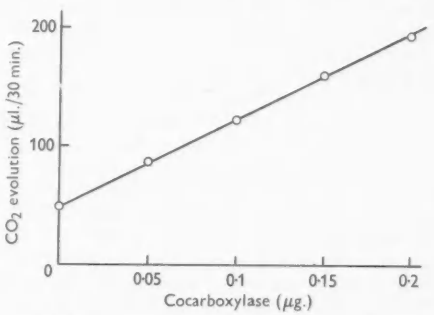


Fig. 1. Relationship between $CO_2$ evolution and quantity of cocarboxylase added to heated blood in a system containing cyanide and enzyme. The evolution at 0·0 μg. is that due to the blood and the enzyme blank.

Cocarboxylase activity is generally lower in reconstituted holoenzyme than in naturally occurring holoenzyme, where it has been found experimentally to be 44 μl. of $CO_2$/min./μg. of cocarboxylase at 30° (Green *et al.* 1941). We have found that the internal-standard activity can reach this figure when good enzymes are available, but generally the activity is about two-thirds of this value.

In the early stages of the work it was found that a basic system of apoenzyme and cocarboxylase showed very little activity. However, when heated blood was substituted for the synthetic cocarboxylase its coenzyme caused some evolution of $CO_2$, and it was concluded that the apoenzyme was active when certain blood constituents were present. Table 2 shows the effects of various substances (some of which may be present in blood) upon the basic enzyme system without blood. Cyanide had the greatest effect upon the activity of the system and was therefore included in subsequent work. Thiosulphate shows an almost equal effect, but no summation results from a combination of these activators. Melnick & Stern (1940) have correlated such effects with the presence of copper in the synthetic cocarboxylase preparation.

When the activity of standard cocarboxylase with cyanide is compared with that of the same standard in the presence of blood and cyanide, the latter is still much higher, demonstrating that some of the blood activators may differ from cyanide in their action (Table 3). To ensure equal activation in tests and standards the internal standard was therefore adopted.

Attempts to produce an ideal external standard by additions of thiamine, citrate and cysteine to the system have failed, because the activity of cocarboxylase, though increased, was still less than that of the internal standard. Plasma, diluted with an equal volume of phosphate buffer, pH 6·2, and added to the 0·05 μg. of cocarboxylase in place of heated blood cells, produced the best approximation to an external standard, since the activity

value was then very close to that of the internal standard used with the corresponding heated blood cells. It seems likely, however, that an external standard based on plasma might vary according to the pathological condition of the subject.

Plasma contains no cocarboxylase, but there is a small amount of thiamine which may exert an activating effect on cocarboxylase. A similar amount of thiamine is also present in the heated cells, and this may cause synthesis or activation of cocarboxylase. Thus what is actually measured in this method is the sum of cocarboxylase and addition of iodoacetate would cause complications through competition by iodoacetate for the various sulphydryl groups available. Thus when blood proteins are present iodoacetate inhibition of cocarboxylase is not always shown, although the phenomenon is quite definite in the simple enzyme system (Stoppani *et al.* 1953).

Another question that arose in the course of this work (cf. Murfitt, 1954) concerned the possibility of a direct reaction between iodoacetate and thiamine, which has been shown to exist in a sulphydryl form (Zima & Williams, 1940). The isolation by Myrbäck, Vallin & Magnell (1945) of

Table 3. *Comparison of the effect of cocarboxylase, in the presence and absence of blood*

Cocarboxylase (1 μg.) was used as (*a*) an external standard, i.e. without blood; (*b*) an internal standard with heated blood. In both (*a*) and (*b*) the enzyme control consisted of apocarboxylase, pyruvate and cyanide. In all flasks the final volume was adjusted to 3 ml. with phosphate buffer.

| | Evolution of $CO_2$ (μl./30 min.) | |
|---|---|---|
| System | Total | Extra due to 1 μg. of cocarboxylase |
| (*a*) Enzyme control | 16 | — |
| Control + cocarboxylase (1 μg.) | 19 | 3 |
| (*b*) Enzyme control + heated blood | 45 | — |
| Control + heated blood + cocarboxylase (1 μg.) | 85 | 40 |

thiamine, but this is still, usually, an indication of the nutritional state of the subject. In the blood of people not receiving therapeutic thiamine, the effect of the free thiamine in the cells is very small. Where large doses of thiamine are given, it is probable that cocarboxylase values are anomalously high because of synthesis from or activation by the free thiamine of the blood. Differential estimation of thiamine and cocarboxylase was attempted by using iodoacetate, but the results were not conclusive.

*Blood cocarboxylase levels in human subjects.* Our results from 105 patients of both sexes who showed no clinical evidence of thiamine deficiency give a normal range of 5–12 μg./100 ml. of blood, with a mean of $7{\cdot}0 \pm 1{\cdot}7$ μg./100 ml. Patients with clinical signs of thiamine deficiency gave values below this range before, and within the range after, thiamine administration. Goodhart & Sinclair (1939) gave the normal range as $7{\cdot}0 \pm 2{\cdot}1$, and Westenbrink & Steyne-Parvé (1949), by their blood-extraction method, gave a range higher than this ($11{\cdot}2 \pm 1{\cdot}5$ μg./100 ml.). Other normal ranges are given by Smits & Florijn (1949).

## DISCUSSION

Activation of cocarboxylase by heated blood cells or plasma is probably due partly to the sulphydryl compounds present. Considering also the sulphydryl nature of carboxylase, it is clear that the the disulphide form of thiamine by oxidation with $H_2O_2$ at pH 7·5 suggests that this form may exist in significant quantity at physiological pH values. It therefore seems possible that iodoacetate and thiamine could react directly and that this could be a contributory explanation of the opposite effects of these substances in the carboxylase system.

The sulphydryl form of cocarboxylase could supply the site of reaction between pyruvate and holocarboxylase, where there is a sulphydryl group which may be reversibly blocked by iodoacetate and protected by pyruvate (Stoppani *et al.* 1953). At present, the intermediate steps in decarboxylation of α-keto acids are little understood, but in all cases the specific configuration of cocarboxylase is required (Ochoa, 1951). Speculation about the place of a thiazole–thiol equilibrium for cocarboxylase in decarboxylation, and the question as to whether or not this furnishes the sole, vital sulphydryl group of holocarboxylase, is of interest. Comparison of such a system for simple decarboxylation with the lipothiamide oxidative decarboxylation system, present in some organisms (Reed, 1953), may help the understanding of this problem.

## SUMMARY

1. A method for the estimation of blood cocarboxylase is described.

2. Innovations include the use of Norite charcoal to obtain a blank-free apoenzyme, the addition of

cyanide to the system, and the use of an internal standard during assay.

3. Plasma showed an activating effect on cocarboxylase.

4. Reference is made to the effects of thiamine and iodoacetate in the system.

5. The possible significance of the sulphydryl form of cocarboxylase in decarboxylation is discussed.

We are grateful to Roche Products Ltd. for a timely gift of pure cocarboxylase.

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# The Effect of pH on the Toxicity of Fluoroacetic Acid

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(*Received* 9 *September* 1955)

The mechanism of the toxic action of fluoroacetate provides one of the few examples of what has been called 'lethal synthesis' (Peters, 1952). Fluoroacetate is not toxic to enzymes (Bartlett & Barron, 1947), but its incorporation into the reactions of the Krebs cycle in place of acetate results in the production of a fluorinated tricarboxylic acid (in all probability, fluorocitric acid) which blocks the action of the enzyme aconitase (Peters, 1952). Since the reactions of the Krebs cycle are associated with the mitochondria of the cell (Nossal, 1953), the action of fluoroacetate must be an intracellular one.

For a number of years a project has been under way in this Laboratory concerning the interactions of the yeast cell and its environment, particularly where changes in pH are involved. The programme is based upon the premise that if the intracellular action of an ionizable chemical agent is found to vary with extracellular pH, then something can be learned about the internal environment of the cell. Iodoacetic acid was first applied to this problem and here pH was found not only to influence the degree of toxicity to yeast cells, but also to determine the characteristics of the inhibitory action observed (Aldous, 1948). The disadvantage of using iodoacetic acid was that its *in vivo* action was characterized by the inactivation of a variety of enzymes (Aldous, 1952). Fluoroacetic acid (HFA), on the other hand, possesses some pharmacological properties which would appear to make it a more suitable agent. This possibility, together with the object of establishing the effect of pH upon the toxicity of HFA to yeast cells, were the reasons for undertaking the present investigation. Yeast cells have been used as the biological material because they will tolerate wide fluctuations in environmental pH without showing appreciable changes in their respiratory metabolism (Barron, Ardao & Hearon, 1950).

A search of the literature showed that whereas a few investigators have noted an effect of pH upon the toxicity of fluoroacetate, none, with the possible exception of Black & Hutchens (1948), has made a systematic study of the phenomenon. These authors noted that as the pH of the medium was lowered from 6·0 to 4·0, the fluoroacetate-induced inhibition of the oxygen consumption of yeast cells was progressively increased. This action is attributed to the fact that for 'fluoroacetic acid ($pK_a$ 3·81) a change in pH from 6·0 to 4·0 increases the concentration of undissociated fluoroacetic acid molecules 60-fold'. No reference for the $pK_a$ is given, but from this value a $K_a$ of $1{\cdot}55 \times 10^{-4}$ can be calculated. Chenoweth (1949) quotes a value of $21 \times 10^{-4}$ for the $K_a$, which is more in keeping with those for the other halogen-acetic acids, and which yields a $pK_a$ of 2·68. By using Chenoweth's figure,

the percentage dissociation of HFA as a function of pH has been calculated and is presented in Fig. 1. This shows that such a strong acid is completely dissociated above pH 5·0, and at pH 4·0 only 5% of the acid exists in the undissociated form. A similar $K_a$ value ($22 \times 10^{-4}$) is given in a recent review by Haszeldine (1954). It seems unlikely, therefore, that the inhibition of oxygen consumption which Black & Hutchens noted could have been due only to the decreased dissociation of HFA.

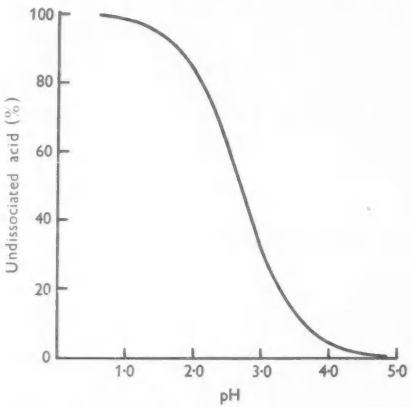


Fig. 1. Percentage dissociation of fluoroacetic acid as a function of pH, calculated from $K_a = 2{\cdot}1 \times 10^{-3}$.

## EXPERIMENTAL

*Material.* The test organism used in all the work reported was the commercially available Fleischman's baker's yeast which was purchased in cake form. The cells were freed from the starch 'filler' by repeated washing and centrifuging. A 1% suspension of cells in 0·05M-$KH_2PO_4$ containing 5% glucose was aerated for 2 hr. Immediately before using them, the cells were centrifuged down and resuspended in citric acid–phosphate buffer (McIlvaine, 1921) of the desired pH, to which glucose was added to make a 5% solution (hereafter referred to as buffer–glucose). The concentration of cells was 1% (v/v).

*Fluoroacetic acid.* Pure sodium fluoroacetate was obtained from Bios Laboratories Inc., New York. This material was dissolved in McIlvaine's buffer and adjusted to the desired pH.

*Viability.* In preliminary experiments, HFA to give final concentrations ranging from $10^{-1}$ to $10^{-4}$M was added to the cell suspension. All exposures were carried out at pH 2·8. The mixture was shaken for 30 min. at 25°, at which time measured samples of the suspension were serially diluted and plated out in wort–agar medium. The colonies arising on these plates after 4 days' incubation at 22° were compared in number with those arising from control (unexposed) cells. All sampling, diluting and plating-out procedures were done in duplicate. These early experiments showed that only when the cells had been exposed to $10^{-1}$M-HFA was there any reduction in colony count; at this concentration the inhibition of cell division was virtually complete.

In later experiments the concentration of HFA was varied from $2 \times 10^{-2}$ to $6 \times 10^{-2}$M with results as shown in Table 1.

Table 1. *Effect of fluoroacetic acid on the viability of yeast cells*

Exposure: 30 min. at pH 2·8.

| HFA concn. ($\times 10^{-2}$M) | 0 | 2 | 3 |
|---|---|---|---|
| Colony counts | 187±20·9 | 197±12·7 | 127±18·0 |
| % of control colonies | — | 105* | 68 |
| HFA concn. ($\times 10^{-2}$M) | 4 | 5 | 6 |
| Colony counts | 22±8·1 | 1 | 1 |
| % of control colonies | 12 | 0·5 | 0·5 |

* Not significantly different from control.

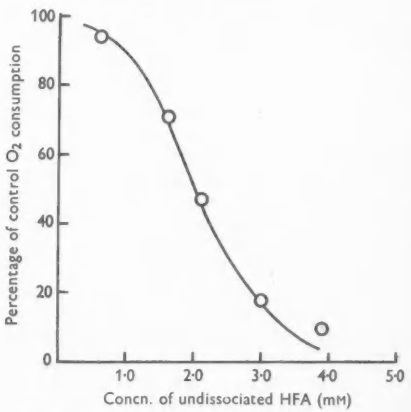


Fig. 2. Inhibition of oxygen consumption of yeast cells by HFA, plotted as a function of the concentration of undissociated HFA in the suspension medium. Concentration of HFA $5 \times 10^{-3}$M; pH varied from 2·15 to 3·50. Duration of experiment 90 min., temp. 25°.

*Oxygen consumption.* The rate of oxygen consumption was measured by the direct method of Warburg at 25°. Exploratory experiments were performed to determine the concentration of HFA which, at a pH close to the p$K$, would produce a 50% inhibition of oxygen consumption. At pH 2·8, this concentration was found to be about $5 \times 10^{-3}$M. With this concentration, the pH of the medium was varied over the range of dissociation of HFA, i.e. from pH 2·0 (the lowest possible with McIlwaine's buffer) to pH 3·5. All control measurements at each pH were made in quadruplicate and all measurements on the HFA-poisoned samples were made in sextuplicate. The results of these experiments are presented in Fig. 2, where the inhibition of oxygen consumption at any given pH is expressed

as percentage of the control rate at the same pH. A number of features not apparent in Fig. 2 should be mentioned: (*a*) the rate of oxygen consumption in the control samples was not influenced by pH; (*b*) the reproducibility in the control samples at any given pH is indicated by the fact that the greatest value of the S.E. was 2·55 for a mean rate of 348 $\mu$l./90 min.; (*c*) the largest S.E. observed in the HFA-poisoned samples was 2·40 for a mean rate of 178 $\mu$l./90 min.

## RESULTS

Since the pharmacological action extends only over the range of dissociation of HFA there can be little doubt that the concentration of undissociated acid is of primary importance in determining the viability or the inhibition of oxygen consumption.

In its influence on viability HFA shows a relatively weak action (Table 1). In order to suppress colony formation completely, a concentration of $5 \times 10^{-2}$M HFA is needed, although it must be kept in mind that the concentration of undissociated HFA here is $2{\cdot}2 \times 10^{-2}$M since the experiments were all carried out at pH 2·8. Viability data in general were difficult to reproduce, and for this reason oxygen consumption was used as a criterion of toxicity in later experiments. It should be noted, however, that from the data of Table 1 calculation shows no reduction in viability until a concentration of $1{\cdot}26 \times 10^{-2}$M undissociated acid is reached. The data for oxygen consumption (Fig. 2) indicate that a 90% inhibition of oxygen consumption is observed when the concentration of undissociated HFA is only $4{\cdot}0 \times 10^{-3}$M. From these considerations it follows that at concentrations intermediate between these two values (e.g. at $1{\cdot}0 \times 10^{-2}$M) a condition must obtain where oxygen consumption will be completely inhibited, yet all cells will be capable of forming colonies. Because serial-dilution and plating-out techniques were used in the viability experiments, it is obvious that reduction in viability represents an irreversible action of HFA; *a priori* it would seem that the observed inhibition of oxygen consumption must represent a reversible action. Attention was thus turned toward an analysis of the reversibility of the HFA inhibition of oxygen consumption.

### *Effect of* pH *on the oxygen consumption of poisoned cells*

Washed and aerated cells were exposed to $5 \times 10^{-3}$M HFA at pH 2·1 for 30 min. at 25°. Measured samples of the suspension, labelled 'unwashed', were pipetted directly into the Warburg vessels. The remainder of the poisoned suspension (as well as the control suspension) was then divided into three portions. One of these was centrifuged and the packed cells were resuspended in fresh buffer–glucose at pH 2·1. After it was stirred thoroughly, the suspension was again centrifuged, and the supernatant was discarded and the cells suspended in buffer–glucose at pH 2·1. The other two portions were put through the washing and resuspending procedures at pH 2·8 and 5·0 respectively. Measured portions of the original control sample were washed and resuspended in buffer–glucose at each of the three pH values. The oxygen consumption of the 'unwashed' and the three 'washed' samples was then followed in the respirometer until a steady state had been reached. Table 2 records the results from one of a series of such experiments.

Table 2. *Reversal of the HFA-induced inhibition of oxygen consumption by* pH

Cells originally exposed to $5 \times 10^{-3}$M HFA at pH 2·1 for 30 min.; they were then washed and resuspended at the same or more alkaline pH values.

| Treatment of sample after poisoning by HFA | Oxygen consumption ($\mu$l./15 min.) Control | + HFA | Percentage inhibition |
|---|---|---|---|
| Unwashed (pH 2·1) | 55·1 | 1·5 | 97·3 |
| Washed at pH 2·1 | 53·5 | 14·2 | 73·5 |
| Washed at pH 2·8 | 53·0 | 24·4 | 54·0 |
| Washed at pH 5·0 | 58·9 | 56·3 | 4·4 |

These data show clearly that the inhibitory action of HFA upon oxygen consumption is freely reversible, provided that the pH of the fluid with which the cells are washed is close to 5·0. The data also confirm the *a priori* assumption made earlier with respect to the action of high concentrations of HFA, for in carrying out the viability experiments, the cells after exposure to HFA were serially diluted through three changes of 0·05M-$KH_2PO_4$ (pH 4·5), and were finally plated out on wort–agar medium (pH 4·8). Thus any inhibition of oxygen consumption, which might have resulted from an exposure of the cells to concentrations of HFA as high as $1{\cdot}0 \times 10^{-2}$M, would be reversed during the dilution and plating-out procedures.

The fact that the degree of reversibility by washing was dependent upon the pH of the washing fluid suggested further experiments. First, the effectiveness of the washing procedure was determined by measuring the oxygen consumption of poisoned cells which had been washed one, two, three and four times in buffer–glucose at each of the pH values in Table 2. The results showed conclusively that a single washing was sufficient to bring about any recovery of oxygen consumption which could occur, and that subsequent washings did not change this new rate.

Secondly, since in the experiments reported in Table 2 the cells were poisoned at one hydrogen-ion

concentration only, it was necessary to know to what extent recovery would occur when the cells were exposed at other pH values. Cells were therefore exposed to $5 \times 10^{-3}$ M HFA at pH 2·2 (no. 1), 2·8 (no. 2) and 3·5 (no. 3). Rates of oxygen consumption were then determined on each of the original samples, and on each sample washed twice as follows: no. 1 at pH 2·2, 2·8 and 4·0; no. 2 at pH 2·8, 3·5 and 4·0; no. 3 at pH 3·5 and 4·0. Table 3 shows the results of this series of experiments in which each value represents at least four observations. In all cases there is a recovery of oxygen consumption upon washing, and the extent of recovery depends upon the pH of the washing fluid. These experiments extend the data presented in Table 2.

Table 3. *Inhibition of oxygen consumption resulting from a 30 min. exposure of yeast cells to* $5 \times 10^{-3}$ M *HFA at various* pH *values followed by a washing at the original or more alkaline* pH *values*

| Sample no. | pH of exposure to HFA | Percentage inhibition of $O_2$ consumption | | | | |
|---|---|---|---|---|---|---|
| | | Not washed | pH of washing fluid | | | |
| | | | 2·2 | 2·8 | 3·5 | 4·0 |
| 1 | 2·2 | 97 | 74 | 58 | — | 27 |
| 2 | 2·8 | 67 | — | 32 | 25 | 16 |
| 3 | 3·5 | 7 | — | — | 0 | 0 |

Table 4. *Inhibition of oxygen consumption of yeast cells exposed to* $5 \times 10^{-3}$ M *HFA at* pH 2·2 *and submitted to various washing procedures*

| Sample no. | pH of washing fluid | pH of final suspension medium | Percentage inhibition of $O_2$ consumption | |
|---|---|---|---|---|
| | | | Expt. *A* | Expt. *B* |
| 1 | 2·2 | 2·2 | 79 | 61 |
| 2 | 2·8 | 2·8 | 59 | 51 |
| 3 | 3·5 | 3·5 | 33 | 34 |
| 4 | 3·5 | 2·8 | 61 | 52 |
| 5 | 3·5 | 2·2 | 56 | 61 |

At this point in the investigation it became quite clear that there were two types of recovery: (1) some could be obtained by merely washing the cells without changing the pH of the environment, and (2) further change could be brought about only by washing the cells at a pH higher than the original. The likelihood that this reversibility was due to dissociation of the inhibitory agent suggested the possibility of re-establishing the inhibition by placing the alkaline-washed cells back into a more acid environment. Experiments were accordingly designed as follows. Cells were exposed to $5 \times 10^{-3}$ M HFA at pH 2·2 for 30 min. The stock suspension was then divided into five portions: no. 1 was washed twice at pH 2·2, no. 2 at pH 2·8, and nos. 3–5 were all washed at pH 3·5. The oxygen consumption of no. 1 was determined at pH 2·2, no. 2 at pH 2·8 and no. 3 at pH 3·5. The respiratory activity of nos. 4 and 5 was measured at pH 2·8 and 2·2 respectively, and thus represented the situation where the cells had been changed from an alkaline back to an acid environment. Control suspensions were subjected to identical washing procedures. The results of two experiments (*A* and *B*) are reported individually in Table 4, where the rate of oxygen consumption of each poisoned sample is expressed as percentage of its respective control rate.

From these results it is clear that the inhibition which is apparently removed by alkaline washing can be made to reappear if the cells are returned to an acid environment. The degree to which this phenomenon occurs is dependent upon the extracellular pH, and takes place within the range of dissociation of HFA.

## DISCUSSION

In interpreting the results relating to the reversibility of the HFA-induced inhibition of oxygen consumption, it is important to note that the phenomenon occurs in what must be an HFA-free extracellular environment. The effect observed must be due either to a binding of the toxic agent to the surface of the cell or to an action within the cell.

Let us examine first the possibility of a surface action. Since toxicity increases with increasing acidity, the action must be due to the molecule of HFA which becomes bound to the cell surface; when the HFA is predominantly in the ionic form there is no inhibitory action (Fig. 2). When the

washed cells are placed in a more alkaline medium, the bound molecules will dissociate into the inert ions, but these must remain attached to the cell in order to account for the data of Table 4. On theoretical grounds the assumptions made in this explanation are highly unlikely. First, HFA is an extremely unreactive compound and, unlike the other halogen-acetic acids, shows no combination with a substance such as cysteine (Chenoweth, 1949). Secondly, it is difficult to explain why the ions resulting from the ionization of the bound molecules should not be repelled by the cell surface and later be removed by washing, or why they should not behave in the same manner as the ions in the original HFA solution. Cells which have been exposed for 30 min. to $5 \times 10^{-3}$M ionized HFA (pH 6·0), and which have then been washed and placed in buffer at pH 2·8, show no inhibition of oxygen consumption. It is thus not possible to 'attach' the HFA in the ionic form to the cell surface. This surface explanation, although consistent with the experimental results, involves an action of the fluoroacetate ion which cannot be shown experimentally, and which is contrary to what might be expected on *a priori* grounds.

If, as an alternative explanation, it is assumed that penetration of the cell wall or membrane takes place, then it appears most likely that the undissociated HFA is the species which penetrates. Once the molecule is inside the cell, the internal pH will determine the extent to which ionization takes place. At once two possibilities arise. The first is that the internal pH is buffered at a value close to 6·3, as reported by Conway & O'Malley (1946). In this situation the molecules of HFA which have entered the cell will become completely ionized, and the intracellular-ion concentration will be determined by the extracellular molecular concentration. The data of Fig. 2 might thus be interpreted as an intracellular action of the fluoroacetate ion. If this ion were free to traverse the cell membrane in an outward direction then washing should remove all the toxic material from the cell regardless of whether the extracellular pH was changed. Experiment shows, however, that recovery does not take place in this manner: washing without changing the pH by no means completely removes the inhibitory effect, whereas washing at an alkaline pH does. If the internal environment is buffered, changes in the external environment should have no influence upon intracellular events, whether the ion were free to pass out of the cell or not. This explanation will therefore account for the data of Fig. 2, but is not consistent with the results presented in Tables 2–4. The second possibility is that the internal environment (or a portion of it, such as Conway's 'outer metabolic region') is free to fluctuate in pH with the external environment. In this case the HFA which had entered the cell in molecular form would ionize to an extent determined by the extracellular pH. Variation in the extracellular pH should vary the concentration of the molecule relatively to that of the ion. Since alkaline washing decreases the inhibition of oxygen consumption, presumably the original equilibrium $HFA \rightleftharpoons FA^- + H^+$ would be displaced to the right, resulting in the formation of inert ion at the expense of the active molecule. The ions so generated would not be able to leave the cell, so that when the cell was returned to an acid environment, active molecules would be regenerated and the inhibitory action would reappear.

The results of Table 4 lead to another important conclusion, namely that the molecule of HFA is unable to leave the cell once it has entered. The explanation for this might lie in the possibility that HFA is metabolized to some intermediate which is both inhibitory and non-diffusible. It would be tempting to ascribe the identity of this toxic intermediate to fluorocitric acid, for two reasons: (*a*) Conway & Downey (1950) have shown that di- and tri-carboxylic acids penetrate the cell very slowly, whereas acetic acid penetrates very rapidly; (*b*) the data of Peters, Wakelin & Buffa (1952) indicate that the p$K$ values of the fluorinated metabolite of HFA should be more acid than those of citric acid; these values in the acid range lie so close to that of HFA that it would be almost impossible, from physiological data, to distinguish between the ionization of HFA and fluorocitric acid.

HFA would presumably enter the cycle in the same manner as acetate does, namely, by conversion into fluoroacetyl coenzyme A, which would then condense with oxaloacetate to form fluorocitrate, following the proposal of Brady & Stadtman (1955).

If the mechanism of action of HFA does in fact involve a 'jamming' of the Krebs cycle in the manner described, and if ionization of the inhibitory agent is not the factor responsible for the reversal by alkaline washing, there is left the seemingly unreasonable assumption that alteration in pH produces a change in the metabolic reactions of the cell. From the data of Fig. 2 this leads to the conclusion that on the acid side of pH 4·5 glucose was being metabolized via the Krebs cycle, while at more alkaline conditions it was not.

Although the yeast cell has been shown to be capable of carrying out all the reactions of the Krebs cycle, Weinhouse & Millington (1947) and Foulkes (1951) have noted features which distinguish it from the cycle as it occurs in animals and higher plants. Both Krebs, Gurin & Eggleston (1952) and Roberts, Cowie, Britten, Bolton & Abelson (1953) are of the opinion that the cycle does not function as a major energy-giving system,

especially when the cell is in the resting condition. From work with *Escherichia coli* Roberts *et al.* (1953) conclude: 'the interpretation of the data shows that the Krebs cycle is of importance in amino acid synthesis, accounting for more than 50 percent of the protein carbon, but relatively unimportant as a mechanism for oxidizing glucose'.

It has been clearly shown by Conway & Brady (1950), and by Rothstein & Enns (1946), that succinic acid is extruded by the resting cell when glucose and potassium ions are present in the external medium. This would suggest that under these conditions the cell only partially utilizes the Krebs cycle. There is also the possibility that in presenting the cell with acetate or any of the other component substrates of the cycle, concentrations far in excess of those occurring under physiological conditions are involved. Thus, although acetate is readily metabolized by yeast cells, it exerts a pronounced inhibitory action upon the utilization of glucose (Maesen, 1953). The significance of such findings must be interpreted with caution, however, because both the rate of oxidation of acetate and its inhibitory action on glucose utilization are materially influenced by both concentration and degree of ionization (Maesen, 1953; Aldous, unpublished results).

It is clear from the foregoing discussion that an answer to the questions raised by the present investigation might be found if more were known about the conditions which set the Krebs cycle in operation. An obvious way to obtain this answer would be to measure the ability of the HFA-poisoned cell to utilize those components of the cycle to which it is normally permeable. Work along these lines is now being done in this Laboratory and will form the subject of a later paper.

The concept that the inhibitory agent may be a metabolite of HFA is an economical one, as it offers a possible explanation for those data of Table 3 which indicate that washing the cells without changing the pH always results in a partial recovery of oxygen consumption. It might be presumed that in the 'unwashed' samples the inhibition was due to the presence of both the inhibitory metabolite and HFA, and that of the washed samples was due only to the former. Washing without changing the pH would thus remove the unmetabolized HFA, which, like acetic acid, might reversibly inhibit the utilization of glucose (Maesen, 1954).

Analysis of the findings of Black & Hutchens (1948), mentioned in the introduction, makes it seem likely that the effect which these authors were observing was one of pH upon the dissociation of the substrate undergoing oxidation. Acetic acid has a $pK_a$ of 4·75 which is near the middle of the range over which the oxygen consumption was measured. From pH 5·0 to 6·0 the concentration of HFA will be unchanged, but there will be a considerable change in the concentration of acetate ions, a factor which appears to be determining the rate of oxidation. The system which these authors were studying thus contained two variables which were likely to change with pH, and it was for this reason that glucose was used as the oxidizable substrate in the present work.

## SUMMARY

1. When yeast cells are exposed to high concentrations of fluoroacetic acid (HFA) in the presence of glucose, the loss in viability which results is proportional to the concentration of undissociated HFA.

2. The inhibition of oxygen consumption which is complete at much lower concentrations of HFA is likewise dependent upon the amount of undissociated HFA in the cell's environment.

3. The inhibition of oxygen consumption is only partially reversed by washing with buffer of the same pH as that at which the cells were exposed to the HFA; this fact suggests that the ultimate inhibitory agent is some non-diffusible metabolite of HFA.

4. The inhibition of oxygen consumption is pH-labile, which indicates either that the inhibitory agent is dissociable, or that the metabolic reactions of the cell change with variation in environmental pH.

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# A Laboratory Electrodialyser and Desalter

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(*Received* 1 *September* 1955)

Electrodialysers described in the literature are for the most part unsuitable for small-scale laboratory use, and details of their performances are not given. Various electrolytic desalters are available which employ a circulating mercury cathode in conjunction with a collodion anode membrane; although widely used in biochemical laboratories they possess several disadvantages. Desalting of a commercial meat extract in such an apparatus was lengthy and resulted in much frothing and evolution of ammonia, owing to decomposition of some of the constituents; finally, the brown colour of the extract was removed, leaving a colourless solution. When meat extract was subjected to exhaustive electro-desalting in the present apparatus none of these gross changes was apparent.

## MATERIALS AND METHODS

*Meat extract.* A commercial extract of meat was dissolved in water and dialysed against distilled water with chloroform as a preservative. The diffusates, free from protein, were combined and concentrated. The solution contained 0·1386 g. of dry matter/ml., 15·1 mg. of N/ml., and 33% of ash.

*Nitrogen.* This was measured by the semi-micro-Kjeldahl method.

*Dry weight.* This was determined by evaporation to dryness at 105° in Pt dishes.

*Ash.* This was determined by igniting the dried samples at 550° in a muffle furnace.

### *Apparatus*

The dialyser (Fig. 1) consists essentially of three square sections cut from 11 mm. Perspex sheet, each bored out in the middle to form a circular chamber 5 cm. in diameter. Two of the sections are closed at one end with a square sheet of 5 mm. Perspex to form the electrode chambers, and into each of these a 9 mm. thickness of 5 cm. diameter carbon rod is cemented to form a sunken electrode 2 mm. below the surface of the section (Fig. 1). Any dead space is filled with Perspex cement.

Holes are bored at each of the four corners, and the three sections when assembled form a block which is held together by wing nuts running on threaded rods passing through the holes. The electrode chambers have holes bored vertically, top and bottom, into which B7 standard ground-glass joints, or cones, are inserted for connexion to the water supply. Communication to the electrode chamber is by drilled-out slots inclined to the membrane in such a way that the water flowing in strikes the membrane, flows across its surface, and flows out through a similar slot, thus reducing stagnation and polarization at the membrane surface.

The centre chamber has a 7 mm. diameter hole in the top. Additional centre chambers are fabricated in the same manner.

Electrical connexion to the electrodes is by short lengths of thin copper rod passing through the plastic and forced into the carbon.

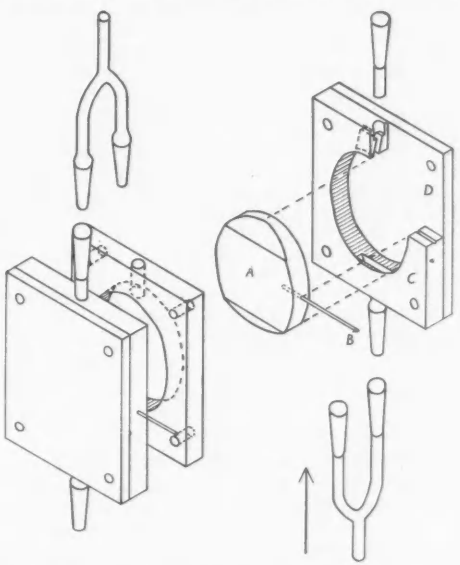


Fig. 1. Isometric view of the electrodialysis cell with one centre compartment in position arranged for desalting. The membranes and the rods and wing nuts are not shown. *A*, carbon electrode 0·9 cm. × 5 cm.; *B*, copper rod; *C*, chamber cut from 1·1 cm. thick Perspex sheet; *D*, Perspex sheet 0·5 cm. cemented to *C*.

*Membranes.* The membranes are held between the ground faces of the component sections and can be of any suitable material. If there are signs of leakage silicone grease may be applied, or rubber gaskets can be inserted.

Two types of membrane have been tested: ordinary collodion dialysis membrane, and Permaplex ion-exchange membranes (The Permutit Co., London, W. 4). Collodion membranes are suitable for removing smaller molecular

species from protein or polysaccharide solutions by electrodialysis. The Permaplex ion-exchange membranes are permeable to inorganic ions and organic ions of size at least up to that of the amino acids, and they are suitable for desalting and electrodialysing non-colloid substances.

*Power supply.* A direct current of 1000 mA at 20 V was applied at the start of the run, and later, as the resistance of the cell increased, the voltage could be increased to 300 with a corresponding current of 100 mA. The area of the membranes is about 20 cm.$^2$, so that current densities up to 50 mA/cm.$^2$ are effective.

*Assembly of the apparatus.* Tap water is passed through the electrode compartments when desalting, but distilled water is necessary for fractionation by electrodialysis.

When the apparatus is arranged for desalting, one centre compartment is used with a Permaplex anion-exchange membrane next to the anode compartment and a similar cation membrane next to the cathode compartment. To check that the membranes are correctly arranged the centre compartment is filled with distilled water and the current is switched on; there should be a small initial flow of current, dropping rapidly to zero. If, however, the membranes have been reversed, there will be a flow of current gradually increasing from a small initial value as the ions in the tap water are driven through the membranes into the centre compartment.

For electrodialysis extra compartments are added and the membranes are arranged in the sequence: cathode, anion membrane, cathode compartment, cation membrane, sample compartment, anion membrane, anode compartment, cation membrane, anode. In this way cations migrating into the cathode compartment are trapped by the anion membrane, and conversely for the cathode compartment. Alternatively, a series of cation membranes can be arranged on the cathode side and a series of anion membranes on the anode side. At the end of the run the farther compartments will contain a higher proportion of the faster-moving components of the mixture.

## RESULTS

### *Preliminary experiments*

The apparatus was assembled for electrodialysis as described, and 0·1 g. of sodium chloride was placed in the centre compartment. A current of 0·90 A was passed, and after 5 min. this had fallen to 0·02 A. The solution was removed and on evaporation no appreciable residue was obtained. The theoretical amount of electricity required to transport 0·1 g. of sodium chloride to the electrodes (assuming that all the current is carried by the ions of the salt) is 166 coulombs. If the average current throughout the run is taken to be 0·90 A, then 270 coulombs were consumed; this represents a maximum value, since the current fell steadily during the run. However, it is clear that the efficiency of desalting was high. Under the same conditions 0·5 g. of sodium chloride could be removed from the centre compartment in 30 min.

When 0·1 g. of glycine was placed in the centre compartment no current could be passed. When the glycine was replaced by 0·1 g. of glutamic acid, the whole of the glutamic acid was transported to the anode compartment in 10 min. at a current of 0·20 A. Conversely, 0·1 g. of arginine was transported completely to the cathode compartment in 5 min. at a current of 0·20 A.

### *Desalting of meat extract*

After preliminary experiments of the type outlined above, a desalting curve for meat extract was constructed.

The meat extract (50 ml.) was placed in the apparatus arranged for desalting. Current was taken from the same tapping of the transformer (50 V) throughout the run. Portions (3 ml.) of the solution were removed for analysis, without affecting appreciably the resistance of the cell at that instant. The dry weight, nitrogen and ash content were determined on each sample and plotted, together with the resistance of the cell, against time. The current fell from an initial value of 0·56 to 0·10 A at the end of the run; the average current was about 0·40 A.

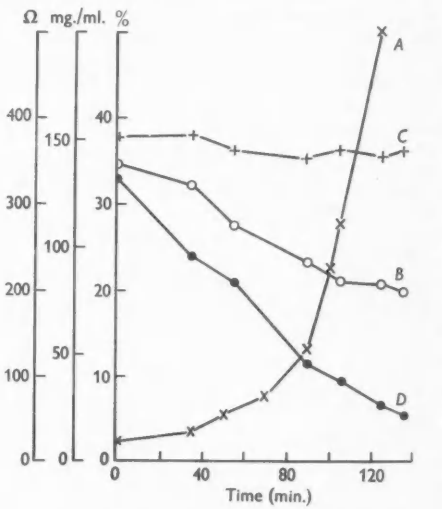


Fig. 2. Desalting of meat extract, *A*, Resistance (Ω); *B*, dry weight (mg./ml.); *C*, nitrogen (mg./ml. × 10); *D*, ash (%).

The curves obtained are shown in Fig. 2. The ash content has fallen from 33 to 5·7%, and the nitrogen content has remained stationary. The sudden steep rise in resistance marks the approaching end of the run when a gradually increasing fraction of the current is being carried by organic

ions. If the run is continued beyond this point, the recovery may fall from 60% of the original material to less than 30%.

Of the 6·93 g. of material taken initially, 3·94 g. (57%) was recovered. Calculated from the initial and final percentages of ash, about 1·87 g. of inorganic material was removed, leaving 16% of the organic material unaccounted for. This figure represents the maximum loss of organic matter, since the exact form in which the inorganic salts were present, before being converted into ash, is not known.

Table 1. *Fractionation of meat extract*

| Cathode compartment | Centre compartment | Anode compartment |
|---|---|---|
| Sodium hydroxide, potassium hydroxide, ammonia, creatine, methylguanidine, trace of carnosine | Carnosine, creatinine, amino acids, creatine, guanidine, carnitine, hypoxanthine, inosine, an unidentified iminazole, a phosphate compound | Hydrochloric acid, phosphoric acid, lactic acid, unidentified organic acids, an unidentified guanidine compound, traces of amino acids |

*Fractionation of meat extract by electrodialysis*

The apparatus was assembled for electrodialysis as previously described; with meat extract in the centre compartment the current was passed for a time considerably longer than that corresponding to the steep rise in the resistance curve (Fig. 2). At the conclusion of the run the contents of each compartment were identified by paper chromatography (see Table 1).

## DISCUSSION

The ease with which any ionic species passes through the membranes will depend on its ionic mobility in the membrane, and in simple systems this can be expressed by its transport number. The transport number of cations in the cation membrane is high and that of anions is low, but the converse is true for the anion membrane (cf. *Permutit Bulletin*, Ion Exchange Membranes). It is upon this fact that the selective permeability of the Permaplex membranes depends. The quantity of electricity carried at any moment by a particular ionic species is proportional to the product $czu$, where $z$ is the ionic charge, $u$ is the mobility, and $c$ is the ionic concentration, which is a function of the molecular concentration and the dissociation constant of the molecule. The fraction of the total current being carried by any particular ionic species depends upon the magnitude of this product relative to the sum of the products for the other ions in solution.

The amount of any substance being transported will therefore depend on its ionic concentration and mobility, relative to the other ions, both in the free solution and in the membrane. In general, these factors operate to favour the separation of inorganic from organic materials. However, electrodiffusion of ions through the membrane can increase the simultaneous diffusion of non-electrolytes (Lightfoot & Friedman, 1954), a factor tending to reduce the efficiency of desalting.

*Practical applications*

It is clearly demonstrated by the recovery and shape of the desalting curves that desalting in this apparatus is efficient, and it should be applicable to a wide range of natural mixtures. Considerable amounts of material, 10 g. or more, can be handled, in the space of a 2 hr. run, so that the apparatus is suitable for preparative work.

The type of fractionation by electrodialysis demonstrated in Table 1 should be a useful preliminary to chromatography, especially where organic acids are to be identified.

## SUMMARY

1. The construction of a simple Perspex electrodialysis cell involving the use of Permaplex ion-exchange membranes or collodion membranes is described.

2. The apparatus can be used for desalting or for fractionation of complex mixtures by electrodialysis.

3. As an example of the use of the apparatus, meat extract containing 33% of ash was desalted to an ash content of 5·7% without appreciable loss of nitrogen.

The same meat extract was separated into an anionic, a cationic and a middle fraction by electrodialysis and the constituents were identified by paper chromatography.

I should like to express my thanks to Dr A. E. Bender and to Dr J. A. Kitchener for advice.

## REFERENCE

Lightfoot, E. N. & Friedman, I. J. (1954). *Industr. Engng Chem.* **46**, 1579.

# Studies on the Enzymic Reduction of Amino Acids: a Proline Reductase of an Amino Acid-Fermenting Clostridium, Strain HF

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(*Received* 3 *June* 1955)

A number of anaerobic bacteria catalyse a coupled oxidation–reduction type of reaction between pairs of amino acids that has come to be known as the Stickland reaction. Proline is a commonly activated electron acceptor in the reductive portion of the reaction, and its product, as shown by Stickland (1935), is δ-aminovaleric acid (reaction 1).

$$\text{Proline} + 2\text{H} \rightarrow \delta\text{-Aminovaleric acid.} \quad (1)$$

Little is known of the mechanism of proline reduction, either at the expense of an oxidizable substrate or by molecular hydrogen. Some evidence that diphosphopyridine nucleotide (DPN) might act as electron carrier was obtained by Mamelak & Quastel (1953), who found that addition of this coenzyme stimulated the interaction of pairs of amino acids in *Clostridium sporogenes*. Also, in agreement with the observations of Nisman & Vinet (1950), they reported the reductive part of the reaction to be sensitive to $5 \times 10^{-3}$ M arsenite, suggesting that an –SH enzyme or coenzyme may be involved.

Experiments described in this paper establish the cofactors necessary for the reduction of proline in enzyme preparations derived from the amino acid-fermenting clostridium, strain HF, and exclude certain pyrroline derivatives as likely intermediates. The discovery that various dithiol compounds are active as ultimate electron donors in the enzymic system has been of considerable aid in obtaining preparations more amenable to detailed observation.

## MATERIALS AND METHODS

The proline reductase system was obtained from lyophylized cells of a clostridium, strain HF, prepared as described elsewhere (Wright & Stadtman, 1956). The cells were ground with alumina according to the procedure of McIlwain (1948) and extracted with 0·01 M-$K_2HPO_4$–$KH_2PO_4$ buffer, pH 7·5. The proline-reductase system in such extracts was found to be stable for at least 2–3 months when stored at −20°. The enzyme withstands prolonged dialysis (42 hr. at 2°) against 0·01 M aminotrishydroxymethylmethane hydrochloride (tris) buffer, pH 8·0, or against 0·01 M-$K_2HPO_4$–$KH_2PO_4$ buffer, pH 7·5 or against 0·01 M-$CH_3.CO_2Na$–$CH_3.CO_2H$ buffer, pH 4·5, without appreciable loss of activity. The enzyme also withstands dialysis against 0·001 M-2:3-dimercaptopropanol (BAL), although the presence of a reducing agent during dialysis was found to be unnecessary.

Enzyme preparations were treated with Dowex-1–HCl or with charcoal (Norite A) for removal of various coenzymes, by the method of Stadtman, Novelli & Lipmann (1951).

Ammonium sulphate precipitation techniques were employed to obtain enzyme fractions enriched in proline-reductase activity. The enzyme solution in 0·01 M phosphate buffer, pH 7·5, was first treated with saturated $(NH_4)_2SO_4$ which was adjusted to pH 3·0 with $H_2SO_4$. For some experiments the protein fraction precipitated at 0·3 saturation and below (fraction A), which contained all of the proline-reductase activity and about one-half of the total protein, was used without further purification. Such preparations were found to be more amenable to further purification, however, if the readily precipitated material was first removed. This left a fraction (precipitated at 0·2–0·3 saturation) containing about 75% of the proline-reductase activity and one-third to one-half of the total protein, and designated fraction B. Occasionally less protein was precipitated at this step, and in some samples of fraction B the proline-reductase system was already purified three- to four-fold over the original extracts. The precipitated protein was dissolved in 0·05 M tris buffer, pH 8·6, and refractionated with solid $(NH_4)_2SO_4$ without further adjustment of pH. The resulting protein fraction (0·3 saturation and below) which contained 25–30% of the protein and most of the enzymic activity of fraction B, is referred to as fraction C. Dialysis of these fractions was unnecessary since no inhibition due to residual ammonium sulphate was noted.

Crystalline ethanol dehydrogenase was a gift from Dr W. Bartley. An acetone-dried powder of sheep-kidney cortex that was used as a source of D-amino acid oxidase was a gift from Dr H. Blaschko, and the L-amino acid oxidase preparation from the digestive gland of *Mytilus edulis* was obtained from Mr D. B. Hope. Generous gifts of BAL, β-mercaptoethanol and 1:3-dithiolpropanol were received from Dr L. A. Stocken (Stocken, 1947), trimethylene- and tetramethylene-dithiol from Dr J. A. Barltrop (Barltrop, Hayes & Calvin, 1954), and reduced 6-thioctic acid (DL-6:8-dithioloctanoic acid) from Dr T. H. Jukes of Lederle Laboratories. D-Proline was kindly supplied by Dr J. P. Greenstein. The [2-$^{14}$C]-DL-ornithine was purchased from Tracer Lab. Inc., of Boston, Mass. DPN was a Pabst Laboratories product of 95% purity.

*Chemical methods.* Proline was measured by the ninhydrin procedure of Chinard (1952) in suitable samples of the reaction mixtures after deproteinizing with ethanol (final concn., 80%, v/v). The various dithiols augment the colour intensity by about 10% when present in the final concentration (0·1 M) and appropriate controls were always

included. The enzymic product, δ-aminovaleric acid, gives no colour with the acid-ninhydrin reagent, even when present at ten times the concentration of proline. For estimation of this product by the Moore–Stein ninhydrin procedure (1948), it was first necessary to separate it from the residual proline by chromatography (Stadtman, 1954).

The thiol concentration of freshly prepared 0·2–0·4M solutions of the various dithiols, and certain of the monothiols, was determined by iodine titration just before use. BAL and 1:3-dithiolpropanol solutions were found to be stable for a few days if stored at −20°.

Protein was estimated by a turbidimetric method standardized against crystalline bovine-serum albumin.

Unless otherwise stated, all enzymic reactions were carried out at 30° in 10 mm. × 75 mm. stoppered test tubes in an atmosphere of $H_2$.

## RESULTS

### *Enzymic reduction of proline to δ-aminovaleric acid*

Evidence that the product of proline reduction in the clostridium HF system is δ-aminovaleric acid was presented in a previous communication (Stadtman, 1954). There is a stoicheiometric relationship under a variety of conditions between the amount of L-proline decomposed and the amount of δ-aminovaleric acid formed with a dialysed crude extract as enzyme source (Table 1). D-Proline and DL-proline were also quantitatively converted into δ-aminovaleric acid. The partially purified enzyme fractions, as well as untreated extracts, attacked both isomers.

*Cofactor requirements for proline reduction.* The cofactors necessary for the reduction of proline are listed in Table 2, which also shows the effect of omitting these substances one at a time from the reaction mixtures. No decomposition of proline in the absence of a suitable thiol was observed and, with an enzyme preparation such as that used in Expt. 2, the reaction was almost completely dependent on the addition of DPN.

Addition of pyridoxal phosphate repeatedly stimulated the reaction by 35–40%, but attempts to achieve greater resolution of the enzyme from this cofactor have so far been unsuccessful. The usual procedures of ageing, incubation of the enzyme at elevated temperatures or irradiation cause such extensive destruction of enzymic activity as to render such treatments impracticable.

The stimulatory effect of magnesium is especially well illustrated by Expt. 4 of Table 2. In Table 3 the data of Expt. *a* show that manganese cannot be substituted for magnesium in the reaction but rather is somewhat inhibitory. The marked inhibition of the system by ferrous iron is illustrated by Expt. *b*.

Table 1. *δ-Aminovaleric acid formation from proline*

L-Proline (2 μmoles) and 1·7 mg. of protein were present initially in each 0·4 ml. sample.

| Sample | Thiol | –SH (M) | Gas phase | L-Proline decomposed (μmoles) | δ-Aminovaleric acid formed (μmoles) | Yield (%) |
|---|---|---|---|---|---|---|
| 1 | BAL | 0·1 | $H_2$ | 0·95 | 1·12 | 118 |
| 2 | BAL | 0·1 | $N_2$ | 1·18 | 1·13 | 96 |
| 3 | BAL | 0·1 | Air | 0·61 | 0·50 | 82 |
| 4 | BAL | 0·2 | $H_2$ | 1·58 | 1·51 | 96 |
| 5 | Lipoic | 0·075 | $H_2$ | 1·81 | 1·78 | 98 |

Table 2. *Reaction-mixture components necessary for proline reduction*

Complete reaction mixtures contained 0·013 μmole of pyridoxal phosphate, 3 μmoles of $MgSO_4$ and 0·2 μmole of diphosphopyridine nucleotide (DPN) in total volumes of 0·4 ml. Also present were: In Expt. 1, 20 μmoles of tris, pH 8·6; 4 μmoles of DL-proline; 20 μmoles of 1:3-dithiolpropanol and 0·5 mg. of protein (charcoal-treated, fraction C). In Expt. 2, 20 μmoles of veronal–carbonate buffer, pH 9·8; 2 μmoles of L-proline; 9 μmoles of 1:3-dithiolpropanol and 0·48 mg. of protein (fraction B). In Expt. 3, 24 μmoles of tris, pH 8·6; 2 μmoles of L-proline; 10 μmoles of BAL and 3 mg. of protein (Dowex-treated extract). In Expt. 4, same as Expt. 3 except 3·3 mg. of protein (dialysed extract).

| Omission | Proline decomposed (μmoles) in 60 min. in Expt. no. 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| None | 2·53 | 1·60 | 0·81 | 0·47 |
| Pyridoxal phosphate | 1·62 | — | 0·52 | 0·32 |
| $Mg^{2+}$ | 1·59 | — | 0·43 | 0·12 |
| DPN | 2·22 | 0·14 | 0·26 | 0·51 |
| Dithiol | 0 | 0 | 0·05 | 0 |
| Enzyme | 0 | 0·04 | — | — |

Table 3. *Effect of metal additions on proline reduction*

Experimental conditions as in Expt. 1, Table 2.

| Metal additions (μmoles) $MgSO_4$ | $MnSO_4$ | $FeSO_4$ | Proline reduced (μmoles) Expt. *a* | Expt. *b* |
|---|---|---|---|---|
| 0 | 0 | 0 | 1·59 | 1·77 |
| 3 | 0 | 0 | 2·53 | 1·62 |
| 0 | 3 | 0 | 1·28 | 1·54 |
| 1·5 | 1·5 | 0 | 1·49 | 1·63 |
| 0 | 0 | 1 | — | 0·60 |
| 3 | 0 | 1 | — | 0·69 |

There appears to be no inorganic phosphate requirement in the proline-reductase system, since enzyme preparations that had been dialysed extensively against 0·01 M tris buffer, pH 8·0, exhibited the same activity in the absence as in the presence of added phosphate.

*Enzymic characteristics of proline reduction.* The optimum pH for proline reduction was found to be on the alkaline side of neutrality for all preparations examined. Enzyme fraction A was most active at pH 9·8 with BAL as the reducing agent. At pH 10·3 it was only about 10% less active, whereas below pH 9·8 the amount of proline reduced decreased gradually and was 40% lower at pH 8·6. There was a downward trend in pH optimum with further purification of the enzyme system and with 1:3-dithiolpropanol rather than BAL as the reducing agent. Thus the optimum pH was about 8·6 for enzyme fraction C (Table 4).

Table 4. *Effect of* pH *on proline reduction*

Reaction mixtures (0·4 ml.) contained 0·01 M indicated substrate, 0·05 M 1:3-dithiolpropanol, and 0·37 mg. of protein. Tris buffers (0·05 M) were employed for pH values 7·6–8·6 and 0·05 M-$NaHCO_3$–$Na_2CO_3$ buffers for pH 9·2 and 9·5.

| | Proline decomposed (μmoles) in 60 min. at pH | | | | |
|---|---|---|---|---|---|
| | 7·6 | 8·2 | 8·6 | 9·2 | 9·5 |
| D-Proline | 2·21 | 3·20 | 3·03 | 2·74 | 2·14 |
| DL-Proline | 1·10 | 2·45 | 2·57 | 1·92 | 1·80 |
| L-Proline | 1·10 | 2·09 | 2·18 | 1·83 | 1·51 |

There was no appreciable difference in pH optimum for the two isomers of proline, but activity of this enzyme preparation was considerably greater on D-proline than on L-proline or the racemic mixture.

The specificity of the enzyme system with respect to amino acid substrate has not been extensively examined, but hydroxyproline is not reduced. The reduction of glycine to acetic acid (Stadtman, 1955), which clearly is catalysed by another enzyme, will be considered in a separate paper.

The heat sensitivity of the proline-reductase system is much more marked in untreated bacterial extracts than in partially purified preparations. For example, 70% of the L-proline-reductase activity of a crude extract was destroyed by heating at 41° for 15 min. at pH 7·4. No protective effect was exerted by the presence of substrate during heating. Fraction C, however, could be heated at 41° at pH 7·4 for 45 min. with only 35–40% loss of activity. Considerable suggestive evidence has been obtained from such experiments for the presence of specific enzymes for D- and L-proline possessing different heat sensitivities.

The concentration of substrate (L-proline) necessary for maximum reduction is about 0·05 M or slightly higher (Table 5). In this experiment δ-aminovaleric acid formation rather than proline disappearance was used as a measure of the extent of reaction, since relatively small decreases in the high proline concentration cannot be measured with precision. The assay method for the product of the reaction is too cumbersome for routine use, however, and therefore the majority of the experiments reported in this paper were conducted under conditions (0·01 M substrate) where proline disappearance could be accurately estimated, even though the rate of the reaction is about 20% less than maximum.

There is a linear relationship between amount of proline reduced and enzyme concentration over a fairly wide range (Fig. 1). For routine studies only about 0·4–0·6 mg. of protein having a specific

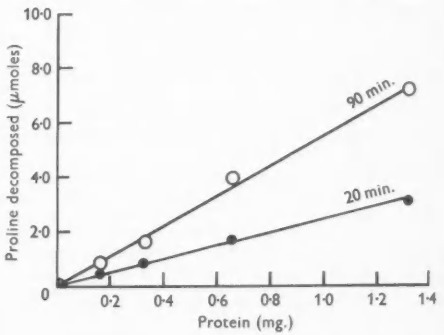


Fig. 1. Effect of enzyme concentration on the reduction of proline. Each 0·4 ml. of reaction mixture contained 0·03 M DL-proline, 3 μmoles of $MgSO_4$, 0·2 μmole of DPN, 20 μmoles of tris buffer (pH 8·6), 20 μmoles of 1:3-dithiolpropanol and indicated amounts of protein (fraction C). Temp. 30°.

Table 5. *Effect of proline concentration on the formation of* δ*-aminovaleric acid*

Each sample contained 0·6 mg. of protein (fraction A), 3 μmoles of $MgSO_4$, 0·2 μmole of DPN, 20 μmoles of 1:3-dithiolpropanol, 20 μmoles of $Na_2CO_3$ buffer (pH 9·7) and substrate as indicated, in a total volume of 0·4 ml.

| | L-Proline concn. (M) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0·005 | 0·01 | 0·02 | 0·05 | 0·075 | 0·1 | 0·2 |
| μMoles of δ-aminovaleric acid formed in 60 min. | 0·79 | 1·15 | 1·1 | 1·34 | 1·44 | 1·44 | 1·17 |

activity comparable with that used for this experiment was employed in 0·4 ml. of reaction mixture. A linear response is also observed with untreated extracts up to at least 1 mg. of protein/0·4 ml. of reaction mixture.

The time course of the reaction under nearly optimum conditions is illustrated in Fig. 2, where it is seen that the rate is linear for incubation periods up to 90 min. For reasons already mentioned the precision of the assays for the shorter

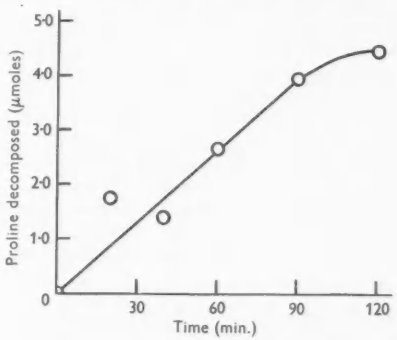


Fig. 2. Each reaction mixture contained 0·66 mg. of protein. Other components as in Fig. 1. Temp. 30°.

incubation times is rather poor. At lower substrate concentrations, where the reaction goes to completion, the rate of proline reduction falls off somewhat towards the end of the incubation period as the substrate becomes exhausted. To obviate this difficulty reactions were normally terminated after 60 min.

*Effect of thiols on proline reduction.* The extracts of clostridium, strain HF, that were employed in the proline-reduction experiments were unable to utilize molecular hydrogen as a reducing agent, although the intact organism does contain an active hydrogenase system. Instead it was found that various thiols would function as electron donors and, as seen in Table 2, the reaction is completely dependent on such additions. A survey of a number of thiol compounds revealed that dithiols, particularly those that are structural analogues of the naturally occurring system 6:8-dithioloctanoic acid (lipoic or thioctic acid), are the most effective. In untreated bacterial extracts or initial ammonium sulphate fractions it has been found, in general, that 6:8-dithioloctanoate, 1:3-dithiolpropanol and trimethylenedithiol are approximately equivalent on a molar basis and are most active. BAL (Fig. 3) and, with some preparations, $H_2S$ are about one-third as effective when tested at comparable thiol levels, whereas β-mercaptoethanol and thioglycollate are essentially inert. Tetramethylenedithiol appears to have some activity, but its very low solubility in aqueous solvents renders it of little use in this work. Cysteine and reduced glutathione fail to activate the proline-reductase system and, moreover, markedly inhibit the complete system containing an active dithiol. The nature of this inhibition has not been determined; however, with reduced glutathione, the inhibition does not appear to be

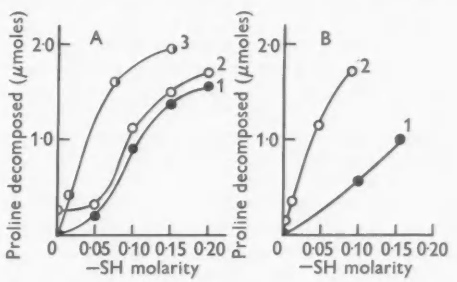


Fig. 3. Effect of thiol addition and degree of anaerobiosis on extent of proline reduction. Reaction mixtures (0·4 ml.) containing 0·005M L-proline, 3 μmoles of $MgSO_4$ and 0·2 μmole of DPN were incubated for 60 min. at 30° in $H_2$. Graph A: samples contained 2 mg. of protein (dialysed crude extract) in 0·05M tris buffer, pH 8·6; curve 1, BAL, 16 mm. × 150 mm. tubes; curve 2, BAL, 10 mm. × 75 mm. tubes; curve 3, DL-6:8-dithioloctanoate, 16 mm. × 150 mm. tubes. Graph B: samples contained 0·48 mg. of protein (dialysed fraction B) in 0·05M veronal–carbonate buffer, pH 9·8; curve 1, BAL; curve 2, 1:3-dithiolpropanol.

due to the formation of oxidized glutathione since the oxidized compound is far less inhibitory when added to the complete system than equivalent amounts of reduced glutathione. It has been observed, also, that the inhibition by glutathione is less marked in partially purified preparations of proline reductase than in the crude bacterial extracts. This observation, when considered together with the fact that crude extracts of clostridium, strain HF, actively decompose both glutathione and cysteine, suggests the possibility that the inhibition is really due to breakdown products. These breakdown products have been detected as characteristic ninhydrin-reactive spots on paper chromatograms. The effects of cysteine on the reaction have been studied less extensively because this compound has the added disadvantage of interfering seriously in the colorimetric proline-assay procedure. Ascorbic acid is inert and borohydride and hydrosulphite inhibit the enzyme system.

Purification of the proline-reductase system was followed with 1:3-dithiolpropanol as the electron donor. When preparations of the type designated as fraction C were then examined for ability to utilize other thiols, it was found that BAL and thioctic acid were now almost inactive, and $H_2S$ was totally so. These results suggest the presence of more than one enzyme in the original extract and in fraction B, responsible for the utilization of the various active thiols (Fig. 3). The wide variation in ability of crude extracts to use $H_2S$ supports the view that this may be one of the more labile enzymes.

The concentrations of the dithiols necessary for maximum proline reduction are extremely high (Fig. 3). The total proline reduced at the lower thiol concentrations is so small as to suggest that the enzyme may not be adequately protected and the reaction may cease at an early stage. That this view is incorrect is shown by the lower curves of Fig. 4; the reaction continues at a slow but fairly constant rate for 90 min. even with 0·023 M thiol.

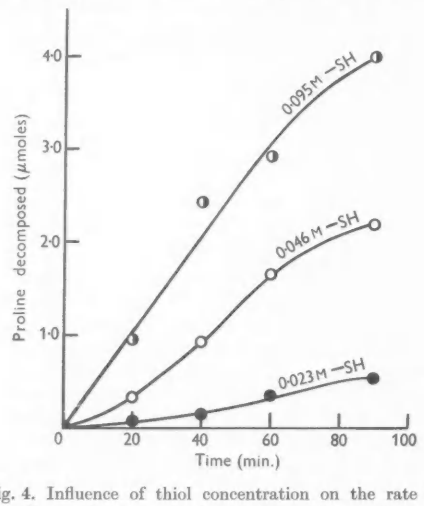


Fig. 4. Influence of thiol concentration on the rate of proline reduction. Each reaction mixture (0·4 ml.) contained 0·8 mg. of protein (fraction C), 0·01 M DL-proline, and 1:3-dithiolpropanol as indicated. Other components as in Fig. 1. Temp. 30°.

The decrease in thiol concentration due to reduction of proline has not been measured because the theoretical ratio of amount oxidized to amount added initially is far too small to detect readily. Also, at the high pH employed, where non-enzymic oxidation of the thiols occurs very readily, there is some loss between the time of addition of the thiol and replacement of the gas phase with $H_2$.

The inhibitory effect of $O_2$ on the reaction is shown in Table 1. Incubation in a stoppered tube with air as the gas phase (sample 3) decreased proline reduction to about half that observed in samples 1 and 2 incubated in $H_2$ and $N_2$ respectively. Even trace amounts of $O_2$ cause a significant inhibition of the reaction, as shown by the observation that in samples incubated in larger-diameter tubes (curve 1, Fig. 3A), which are more difficult to flush efficiently with $H_2$, less proline was reduced than in a parallel series incubated in small-diameter tubes (curve 2, Fig. 3A).

*Effect of reduced DPN on proline reduction.* A dehydrogenase from *Streptococcus faecalis* has been described by Gunsalus (1954) that catalyses the formation of reduced DPN (DPNH) from DPN and reduced lipoic acid. The equilibrium for this reaction appears to be far in favour of DPNH formation. If a similar enzyme is a functional component of the proline-reductase system of clostridium, strain HF, the overall reaction 1 might then be envisaged as the sum of reactions 2 and 3, wherein DPN serves as the intermediate electron carrier:

$$\text{Dithiol} + \text{DPN} \rightarrow \text{Disulphide} + \text{DPNH}, \quad (2)$$

$$\text{DPNH} + \text{proline} \rightarrow \text{DPN} + \delta\text{-aminovaleric acid}. \quad (3)$$

To test this hypothesis, the effectiveness of DPNH as an electron donor in the proline-reductase system was examined. For these experiments, ethanol and ethanol dehydrogenase were added to reaction mixtures containing DPN to generate a continuous supply of DPNH. The activity of the ethanol dehydrogenase in the absence of dithiol, but in the presence of the other components employed in the proline-reductase system, was determined spectrophotometrically, and sufficient enzyme was added to each sample to give the indicated rate of DPN reduction (Table 6). These rates were found to be depressed about 60 % at high levels of dithiol (0·1 M–SH), and therefore the amount of DPNH available for proline reduction may have been less in such samples. It was found, however, that DPNH supplied in this way is incapable of functioning either as an alternative electron donor or as a supplementary electron donor for proline reduction (Table 6). The amount of proline decomposed is no greater in the series of samples to which the DPNH-generating system was added than in the parallel series without ethanol dehydrogenase and ethanol.

*Reduction of proline to δ-aminovaleric acid in the presence of* [5-$^{14}$C]-$\Delta^1$-*pyrroline-5-carboxylic acid.* Since the function of DPN in the proline-reductase system does not appear to be simply that of an electron-transfer agent from thiol to substrate, an alternative possibility that it might be involved in an initial oxidative attack on the molecule,

followed by ring opening and reduction, was tested. If such was the mechanism then one would reasonably expect the formation of $\Delta^1$-pyrroline-2-carboxylic acid as an intermediate which might exchange with free added [$^{14}$C]pyrrolinecarboxylic acid and thus give rise to [$^{14}$C]-δ-aminovaleric acid in the complete system. For this experiment radioactive pyrrolinecarboxylic acid was prepared by oxidation of [2-$^{14}$C]-DL-ornithine with L-amino acid oxidase. Theoretical oxygen uptake for the L-isomer present in 10 μmoles of DL-ornithine (3·9 μc of $^{14}$C) was observed. The pyrrolinecarboxylic acids resulting from spontaneous ring closure of the isolated by means of paper chromatography and examined for radioactivity. In all cases where enzyme was present, the proline had completely disappeared and corresponding amounts of δ-aminovaleric acid had appeared. All samples of the latter, however, were completely free of isotope. In fact, the pattern of $^{14}$C distribution on paper strips was the same for each sample and its control, which indicated that both the [$^{14}$C]pyrrolinecarboxylic acid and the residual [$^{14}$C]-D-ornithine were inert in the purified enzyme fraction. As an additional check, the oxidized product of D-proline was prepared with D-amino acid oxidase and iso-

Table 6. *Effect of the addition of a DPNH-generating system on proline reduction*

Expt. 1. Each 0·4 ml. of reaction mixture contained BAL as indicated, 0·005 M L-proline, 0·05 M tris buffer (pH 8·6), 3 μmoles of $MgSO_4$, 0·2 μmole of DPN, 0·5 mg. of protein (fraction A), and ethanol dehydrogenase where indicated sufficient to reduce 0·03–0·04 μmole of DPN/min. Expt. 2. Each 0·4 ml. of reaction mixture contained 1:3-dithiolpropanol as indicated, 0·01 M DL-proline, 0·8 mg. of protein (fraction C), and ethanol dehydrogenase where indicated sufficient to reduce 0·1–0·2 μmole of DPN/min. Other components as in Expt. 1.

| Expt. no. | –SH (M) | Proline reduced (μmoles): Proline reductase system alone | Proline reduced (μmoles): Proline reductase system plus ethanol dehydrogenase and ethanol (0·1 M) |
|---|---|---|---|
| 1 | 0 | 0 | 0·05 |
| | 0·055 | 0·46 | 0·49 |
| | 0·11 | 1·07 | 1·14 |
| | 0·16 | — | 1·44 |
| 2 | 0 | 0 | 0 |
| | 0·02 | 1·21 | 0·99 |
| | 0·04 | 2·04 | 2·04 |
| | 0·08 | 3·62 | 3·55 |
| | 0·1 | 3·80 | 3·88 |

α-keto acid was isolated by the paper-chromatographic procedures of Vogel & Davis (1952) and Fincham (1953). As described by these investigators, chromatograms developed in 50% (v/v) aqueous ethanol showed a single spot at $R_F$ 0·74, giving a positive Ehrlich's test and containing $^{14}$C. The residual [$^{14}$C]ornithine occupied a lower position on the chromatogram. In *n*-butanol–acetic acid–water (75:4·5:18·5) the product gives two spots of about equal intensity. Only one of these ($R_F$ 0·24) was isolated from the labelled-ornithine reaction mixture, since the lower one ($R_F$ 0·07) occupied the same area as the residual ornithine and could not be detected. Both samples of the isolated product, as well as a portion of the unfractionated [$^{14}$C]ornithine reaction mixture, were incubated in the complete proline-reductase system in which fraction C was used as enzyme source. A control lacking only the enzyme was included for each sample. After termination of the reactions, the δ-aminovaleric acid formed was lated by the chromatographic procedures described above. The same spots giving a positive Ehrlich's test were found as with the oxidized ornithine reaction mixture. The isolated material tested at a concentration equal to that normally used for proline in the proline-reductase system (in the absence of proline) likewise gave no δ-aminovaleric acid. From these experiments it is clear that $\Delta^1$-pyrroline-5-carboxylic acid and its equilibrium product, $\Delta^1$-pyrroline-2-carboxylic acid, are not free intermediates in the reduction of proline to δ-aminovaleric acid.

## DISCUSSION

The proline-reductase system of a clostridium, strain HF, catalyses a quantitative reduction of proline to δ-aminovaleric acid, provided that any one of a number of dithiols is also present to serve as the ultimate electron donor. The high thiol concentrations required for maximum proline

decomposition may indicate merely that the dithiols employed are less efficient analogues of the natural substrates for the enzymes. Of course, the normal coupling in the intact bacterial cell of the oxidation of ornithine with the reduction of proline may occur by a mechanism quite independent of a dithiol substrate or catalyst. The additional requirement for DPN in the system suggested that it might play a role as electron-transport agent between dithiol and amino acid substrate. Indeed, it was found that partially purified preparations of proline reductase that catalysed the reduction of proline by 1:3-dithiolpropanol also contained a DPN-linked dehydrogenase for this dithiol. That part of the observed increase in absorption at 340 m$\mu$. was actually due to DPNH formation was shown by obtaining a 50% decrease in absorption on the addition to the reaction mixture of acetaldehyde and ethanol dehydrogenase. The residual ultra-violet-absorbing material was undoubtedly oxidized 1:3-dithiolpropanol, since the 5-membered S–S ring of this compound also has an absorption maximum in the region of 340 m$\mu$. (Barltrop *et al.* 1954). Although these data indicate that the clostridium preparations have their own mechanism for reducing DPN by a dithiol, the addition of another DPNH-generating system (ethanol and ethanol dehydrogenase) failed to replace the dithiol or supplement it at rate-limiting levels. This was true both when catalytic levels of DPN were added to the system and also when only the amount still bound to the protein of the clostridium enzyme fraction was present. Thus it would appear that the reduction of proline does not occur by a mechanism involving reactions 2 and 3.

Another possible catalytic role of DPN in the system, that of primary electron acceptor for an initial oxidative attack on proline giving rise to $\Delta^1$-pyrroline-2-carboxylic acid or $\Delta^1$-pyrroline-5-carboxylic acid or both, seems unlikely, since these compounds were shown not to be free intermediates in the reduction of proline. Earlier tracer experiments with growing cultures of clostridium, strain HF, also provided suggestive evidence that the ornithine-oxidative and proline-reductive pathways do not have common intermediates, since only a very small amount of ornithine carbon appeared in δ-aminovaleric acid, the product of proline reduction (Stadtman & White, 1954). In this system ornithine oxidation apparently occurs via the α-keto acid which can give rise, non-enzymically, to the pyrroline derivatives mentioned above. Spectrophotometric experiments furnish further evidence against an initial DPN-linked oxidative attack on proline. No increase in absorption at 340 m$\mu$. was seen when the enzyme was incubated with all the essential reaction mixture components except the thiol. Also, attempts to demonstrate the accumulation of some intermediate formed in reaction mixtures containing substrate amounts of enzyme which might subsequently undergo a non-enzymic reduction by the dithiol were unsuccessful. In brief, no reaction has been observed unless enzyme and substrate and thiol were all present simultaneously.

An explanation of the apparent pyridoxal phosphate requirement of the proline-reductase system is also lacking. Both optical isomers of proline are decomposed and therefore it is possible that a racemase is a component of the system, and this might require pyridoxal phosphate as a coenzyme (Snell, 1952). A reaction of pyridoxal phosphate with the ring N of proline is, however, somewhat difficult to visualize. Moreover, the action of certain heated enzyme preparations on D-proline and on L-proline was more suggestive of the presence of an enzyme for each isomer than of a racemase.

Although the enzyme system employed in the present experiments appears to be less complex than those that rely on another amino acid or molecular hydrogen as the electron donor, it has not been possible yet to deduce the mechanism whereby the proline ring is opened and reduced to form the straight-chain product, δ-aminovaleric acid. Further purification of the enzymes responsible for the overall reaction appears to be necessary in order to achieve this aim.

## SUMMARY

1. The proline-reductase system of a clostridium, strain HF, catalyses a quantitative reduction of DL-proline to δ-aminovaleric acid. In unfractionated extracts, a number of thiols serve as reducing agents for the process. Certain dithiols structurally related to 6:8-dithioloctanoic acid (lipoic or thioctic acid) are most active.

2. The cofactors necessary for maximum proline reduction in the isolated system are diphosphopyridine nucleotide (DPN), $Mg^{2+}$ and pyridoxal phosphate.

3. The enzymes responsible for the reduction have been partially purified by ammonium sulphate precipitation. The ability of a number of the thiols to serve as reducing agents is lost on purification of the enzyme system.

4. Although the preparations contain a DPN-linked dithiol dehydrogenase, reduced DPN neither replaces the dithiol nor supplements it at rate-limiting concentrations in the proline-reductase system.

5. $\Delta^1$-Pyrroline-5-carboxylic acid is not a free intermediate in the reduction of proline to δ-aminovaleric acid.

Prof. D. D. Woods generously allotted space in his laboratory for the pursuance of these studies, which were carried out during the tenure of a fellowship grant from the Helen Hay Whitney Foundation.

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# Amino Acid Composition of Elastoidin

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(*Received* 26 *July* 1955)

The name elastoidin was given to the fibrous protein of the ceratotrichia or fin-rays of the elasmobranch, *Mustelus laevis*, by Krukenberg (1885). He noted its similarity to collagen, but was of the opinion that it differed from the latter in essential respects, particularly in not yielding gelatin on boiling with water. Except for an elementary analysis made by Krukenberg, the only chemical study recorded on elastoidin is that by Engeland & Bastian (1938), who qualitatively identified glycine, alanine, serine and hydroxyproline in the acid hydrolysate. However, the material was made the subject of several physical studies (see Champetier & Fauré-Fremiet, 1937; Astbury & Lomax, 1935) and on the basis of its wide-angle X-ray diffraction it was classified as a collagen by Astbury (1938).

Ceratotrichia of selachians afford a convenient material for investigation of collagen because of the simplicity of their anatomical disposition in the fins and the ease with which they can be obtained in homogeneous form. When a shark fin sectioned at the base is split open longitudinally by pulling out the cutaneous sides from each other, the ceratotrichia are exposed as long translucent fibres embedded in the fleshy tissue. If the halves of the fins are soaked in water for some time, the fleshy material is sufficiently softened for the fibres to be pulled out intact. From large fins, fibres reaching 6–9 in. in length and 1–2 mm. in diameter are often obtained in quantity; they are of a translucent light-yellow colour, morphologically homogeneous and sufficiently rigid to be easily washed free from any adhering tissue, the last traces of which can be completely removed by digestion with trypsin.

The amino acid composition of the elastoidin of the fin-rays of *Carcharinus melanopterus* is described here. The amino acid distribution follows the general pattern of typical collagens, but also shows certain significant differences which might explain the peculiarities in physical behaviour observed with elastoidin.

## EXPERIMENTAL

*Preparation of elastoidin.* Elastoidin fibres were isolated from the dorsal fin of the shark *Carcharinus melanopterus*. The fin was obtained fresh within 12 hr. of the catch, split into two halves and soaked for 48 hr. in water under toluene, the water being periodically changed. The fin-rays were pulled out of the fleshy portion of the fin individually and washed free from adhering tissue.

The isolated fibres (12 g.) were immersed in 600 ml. of 2% $NaHCO_3$ solution containing 600 mg. of trypsin (British Drug Houses, Ltd., L.R.) protected by a toluene layer and left for 48 hr. at 37°. The enzyme solution was then poured off and the rays were washed repeatedly with distilled water. The fibres were preserved in water under toluene in the cold. For analysis the fibres were snipped and macerated in a Waring Blendor with water at 0°, and the disintegrated material, consisting of fine fibres, was filtered off, washed with acetone and defatted by Soxhlet extraction with light petroleum (b.p. 40–60°).

### *Analysis*

*Moisture, ash, nitrogen and sulphur.* Moisture was determined by drying to constant weight over $P_2O_5$ at 110° and 1 mm. pressure. The N content of the protein

(corrected for moisture 5·27%, ash 0·73%) was 17·52% (micro-Kjeldahl). S content determined according to the procedure described by Pirie (1932) was 0·51%. Ester-bound S was estimated as sulphate-S after hydrolysis with 5N-HCl for 20 hr. at 100° (Lugg, 1938*a*) and found to be 0·03%.

*Hydrolysis.* The disintegrated and defatted fibre (1 g.) was dissolved on the water bath in 200 ml. of 6N-HCl (redistilled twice in glass) and refluxed for 20 hr. in an oil bath maintained at 135–140°. The excess of HCl was removed by repeated concentration under reduced pressure and finally by leaving over KOH pellets in a vacuum desiccator. The residue was dissolved in 0·5N-HCl and made up to volume with water.

*Chromatographic techniques.* The amino acid separations were carried out by chromatography on Dowex-50 (200–400 mesh, 12% cross-linked) columns operated in the sodium form essentially as described by Moore & Stein (1951). A 1cm. × 100 cm. column was used for quantitative determination of acidic and neutral amino acids and for a qualitative picture of the components in the basic amino acid range. The basic amino acids were estimated in separate runs on a 1 cm. × 15 cm. column. Therate of flow of solvent was maintained by applying a positive or negative pressure to the top of the separating funnel containing the buffer through an empty 10 l. aspirator bottle maintained at the proper pressure. Effluent fractions (1 g.) were collected on a Shandon automatic fraction collector. The effluent fractions were analysed by the photometric ninhydrin method of Moore & Stein (1948), hydroxyproline and proline being estimated at 440 mμ. in the Beckman Model DU quartz spectrophotometer and the other amino acids on a Klett–Summerson photoelectric colorimeter, with a 540 mμ. green filter. Identification of substances responsible for the various peaks was carried out in preliminary column runs by the use of paper chromatograms, after pooled samples of fractions comprising the peak points had been desalted in the way described by Stein (1953).

*Estimation of cystine, methionine and tryptophan.* Cystine was estimated polarographically by the procedure of Stern, Beach & Macy (1939) in samples hydrolysed with HCl–formic acid (Block & Bolling, 1951) under $N_2$. Methionine was determined by the volatile-iodide method of Baernstein (1936), in which the protein was hydrolysed and demethylated simultaneously by boiling under reflux with conc. HI under $N_2$. Tryptophan was estimated by the *p*-dimethylaminobenzaldehyde method of Horn & Jones (1945) in alkali hydrolysates of the protein (Lugg, 1938*b*).

*Ammonia.* The $NH_3$ content of elastoidin hydrolysates was determined by the microdiffusion method of Conway (1947).

## RESULTS

### *Hydrothermal behaviour*

The shrinkage temperature of the elastoidin fibre as measured in the Theis Shrinkage Tester was found to be 63–64° in water, the fibre contracting to about 30% of its original length. On cooling and applying longitudinal tension, the shrunk fibre became re-elongated to about 85% of its initial length. These properties are in accord with the results reported by Fauré-Fremiet (1937).

As observed by this author, prolonged treatment with steam is necessary to get elastoidin into solution. By repeated autoclaving with water at 120° (seven extractions for 4 hr. each) 98% of the material was obtained in solution. The solution on concentration and cooling set to a gel.

### *Optical properties*

Native and re-elongated elastoidin fibres showed strong birefringence under the polarizing microscope, whereas the shrunk fibre had only a feeble birefringence.

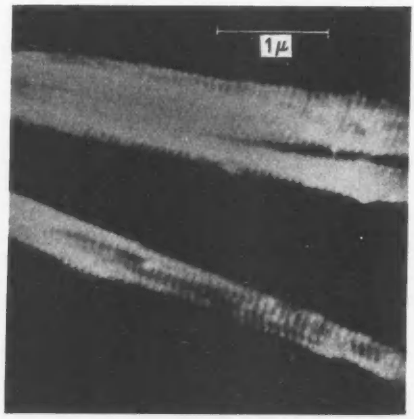


Fig. 1. Electron micrograph of elastoidin fibres treated with trypsin and fixed in formalin. Chromium-shadowed.

For electron-microscopic examination the intact fibres were treated for 48 hr. with trypsin, fixed for 24 hr. in 10% formalin and dispersed in water in the Waring Blendor. Samples were shadowed with chromium at an angle of 20°. Band spacings (Fig. 1) varied between 750 and 880 Å in preparations examined by this method. A periodicity of 600 Å has been reported in dry elastoidin fibrils by Bear (1952) from low-angle X-ray studies.

### *Action of enzymes*

The intact elastoidin fibre resisted the action of crude or crystalline trypsin (British Drug Houses Ltd., L.R. and Armour and Co., cryst.), was slightly acted on by papain (crude latex suspension) and was completely dissolved by crude or crystalline pepsin (British Drug Houses Ltd. and Armour and Co., cryst.). The shrunk fibre was completely digested by all three enzymes.

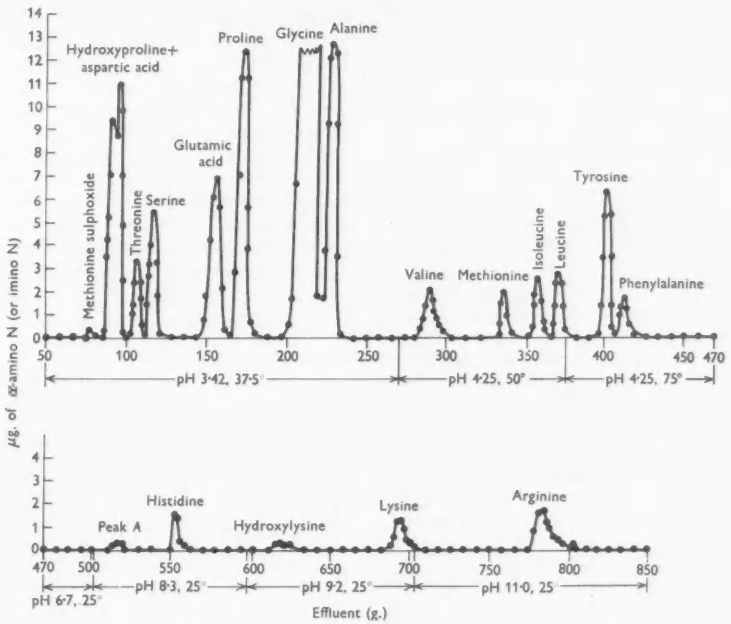


Fig. 2. Separation of amino acids from 5 mg. of elastoidin hydrolysate on a 1 cm. × 100 cm. column of Dowex-50.

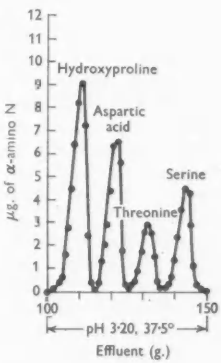


Fig. 3

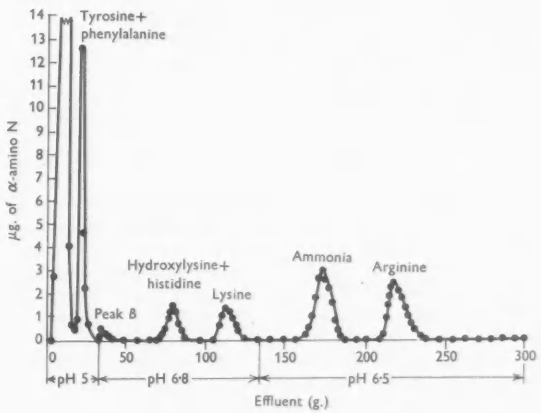


Fig. 4

Fig. 3. Separation of hydroxyproline, aspartic acid, threonine and serine; 5 mg. of elastoidin hydrolysate on a 1 cm. × 100 cm. column of Dowex-50.

Fig. 4. Separation of basic amino acids; 5 mg. of elastoidin hydrolysate on a 1 cm. × 15 cm. column of Dowex-50.

*Amino acid analyses*

Five different types of chromatograms were employed in the analysis of elastoidin hydrolysates. Threonine, serine, glutamic acid, proline, valine, isoleucine, leucine, tyrosine and phenylalanine were estimated on the 100 cm. column with a 5 mg. load and the usual buffer systems (Moore & Stein, 1951). A typical effluent curve is shown in Fig. 2. Hydroxyproline and aspartic acid, which were not well separated at pH 3·42, were resolved by the method of Moore & Stein (1951) by using a buffer of pH 3·20 (Fig. 3). Since the 5 mg. load was too high for satisfactory resolution of glycine and alanine, the two were separated with 1 mg. of hydrolysate. Lysine and arginine were determined on the 15 cm. column with a 5 mg. load (Fig. 4) according to the procedure of Moore & Stein (1951), and histidine and hydroxylysine were separated on the 15 cm. column with a 10 mg. load by the method of Piez (1954). A value of 0·87% on a nitrogen basis was found for hydroxylysine, compared with 0·73% reported by Ramachandran (1953). The ninhydrin-positive peaks, peak *A* (Fig. 2) and peak *B* (Fig. 4), which appear in addition to those expected at the positions of emergence of the common amino acids, could not be identified in paper chromatograms, as the amount of material was too low. The position of emergence in the two columns, however, indicates $\beta$-alanine (Moore & Stein, 1951).

The destruction of serine and threonine was estimated after refluxing known quantities with 6 N-HCl for 20 hr. The losses were found to be 3·5% for serine and 1·0% for threonine. No corrections were applied for these losses in tabulating the analytical values. The stability of serine and threonine to a 22 hr. period of acid hydrolysis has also been reported by Dustin, Czajkowska, Moore & Bigwood (1953).

## DISCUSSION

The results of the amino acid analyses of elastoidin are summarized in Table 1. The composition found exhibits the main features considered characteristic of collagens (Kendrew, 1954). Thus there is a high content of glycine, amounting to about 32%, and of hydroxyproline and proline, together amounting to about 17% of the total residues. Residues of non-polar amino acids total 64%. The hydroxy amino acids hydroxylysine, threonine and serine are present in about the proportions usually found in mammalian collagens (Bowes & Kenten, 1948; Neuman, 1949); the hydroxyproline content is lower, but the quantity is comparable to that found in fish collagen (Neuman & Logan, 1950; Gustavson, 1955). These chemical characteristics explain the molecular arrangement which elastoidin and collagens have in common, as shown by their similar wide-angle X-ray patterns.

A striking deviation from collagen composition is the high tyrosine content of 7·15% by weight, compared with the usual value of about 1% for collagens. Significance is also to be attached to the presence of 0·35% of cystine which is not present in cattle-hide collagen, and according to most analyses is present only in insignificant amounts in other collagens. These two differences in chemical composition should be relevant to the properties that distinguish elastoidin from collagens in general.

Table 1. *Amino acid composition of elastoidin*

| | g./100 g. of protein | N as % of total N | Residues/$10^5$ g. of protein |
|---|---|---|---|
| Alanine | 11·40 | 10·23 | 128·0 |
| Glycine | 25·37 | 27·04 | 338·0 |
| Valine | 2·72 | 1·86 | 23·2 |
| Leucine | 2·62 | 1·59 | 20·0 |
| Isoleucine | 2·69 | 1·64 | 20·5 |
| Proline | 13·27 | 9·22 | 115·3 |
| Hydroxyproline | 8·76 | 5·35 | 66·8 |
| Phenylalanine | 2·10 | 1·02 | 12·7 |
| Tyrosine | 7·15 | 3·16 | 39·5 |
| Tryptophan | 0·00 | 0·00 | 0·0 |
| Serine | 3·31 | 2·52 | 31·5 |
| Threonine | 2·42 | 1·62 | 20·3 |
| Cystine | 0·35 | 0·23 | 1·5 |
| Methionine | 1·78 | 0·96 | 12·0 |
| Arginine | 8·61 | 15·81 | 49·5 |
| Histidine | 1·73 | 2·68 | 11·1 |
| Lysine | 3·74 | 4·09 | 25·6 |
| Hydroxylysine | 0·88 | 0·87 | 5·4 |
| Aspartic acid | 6·40 | 3·85 | 48·1 |
| Glutamic acid | 11·01 | 5·98 | 74·9 |
| Amide N | — | 3·40 | (42·5) |
| Total | 116·3 | 103·1 | 1045·3 |

Lloyd (1941), in comparing the swelling behaviour of collagen, elastoidin and ovokeratin, was of the opinion that with increase in sulphur content these substances showed increasing resemblance to keratin, probably because of disulphide bridges between polypeptide chains. The similarity to keratin in chemical composition, shown by the presence of tyrosine and cystine, may also afford an explanation for the observed hydrothermal properties of elastoidin. Gustavson (1955) advanced the hypothesis that the hydrothermal properties of the collagens depend on the hydrogen-bond linkage of the OH group of the hydroxyproline to the keto imide group of the peptide chain. This was based on the finding that skin collagens of cod fish and pike, which have a shrinkage temperature of 40° and 55° respectively, compared with 65° for bovine collagen, have also lower contents of hydroxyproline (5·8 and 7·9% respectively) than bovine collagen (12·7%). This does not apply to

elastoidin, which has a high shrinkage temperature of about 64°, but a low hydroxyproline content similar to that of fish collagen. The reversibility of thermal shrinkage shown by elastoidin in its natural state also indicates the possibility of the presence in elastoidin of cross-linkages that do not occur in other collagens, since these manifest reversible shrinkage only after treatment with formalin (Gerngross & Katz, 1926). The cross-linking may also afford an explanation for the slowness with which elastoidin is converted into gelatin even under drastic treatment with steam under pressure.

## SUMMARY

1. The amino acid composition of elastoidin from the ceratotrichia of *Carcharinus melanopterus* has been determined.

2. The amino acid distribution follows the general pattern characteristic of collagens, and justifies the inclusion of elastoidin in the collagen group of proteins made on the basis of physical studies.

3. Elastoidin differs from other collagens, being rich in tyrosine and containing a significant amount of cystine. These differences may explain the peculiar hydrothermal behaviour of elastoidin which distinguishes it from other collagens.

We wish to thank Dr M. K. Gharpurey for the electron micrograph and Mr R. K. Agashe for technical assistance.

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# Comparison of the Metabolism of Uniformly $^{14}$C-labelled L-Phenylalanine, L-Tyrosine and L-Tryptophan in the Rat

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(*Received* 26 *August* 1955)

In the adult animal whose metabolism is in the steady state the amount of ingested tryptophan incorporated into body protein should be balanced by liberation of an equivalent amount of endogenous tryptophan by protein breakdown. The observed degradation products of tryptophan should therefore account for an amount of tryptophan equal to that ingested. In fact, as is shown in the subsequent discussion, this is very far from being the case. Our knowledge of the metabolism of tryptophan differs from that of the metabolism of phenylalanine and tyrosine in a most important respect. Phenylalanine is metabolized via tyrosine to the metabolically highly reactive acetoacetic and fumaric acids, so that metabolism of large amounts of phenylalanine and tyrosine can be readily accounted for. But there is no pathway at present known for conversion of the aromatic ring of tryptophan into metabolically reactive aliphatic compounds.

It was hoped that light might be thrown on this problem by comparing the metabolism of uniformly $^{14}C$-labelled L-phenylalanine, L-tyrosine and L-tryptophan in the rat. The results described in this paper show that a fairly readily available pathway (or pathways) for degradation of tryptophan to aliphatic substances must exist, and suggestions are made concerning its nature.

## EXPERIMENTAL

*Animals.* Male white rats weighing between 190 and 210 g. were taken from the School stock as required. A total of eighteen animals was used. They were starved overnight (16 hr.) before administering the amino acid in solution by stomach tube.

*Amino acids.* Uniformly $^{14}C$-labelled L-tyrosine, L-phenylalanine and L-tryptophan were prepared biosynthetically in one of two ways (C. E. Dalgliesh & R. W. Dutton, paper in preparation). In the first procedure uniformly labelled carbohydrate prepared by photosynthesis with tobacco leaves was used as substrate for growing *Torulopsis utilis*. The yeast protein was isolated and hydrolysed enzymically, and the amino acids were separated by column chromatography. In the second procedure direct photosynthesis by *Chlorella vulgaris* was used. The uniformity of labelling was inferred from the biosynthetic procedures, and was not confirmed by degradation. The amino acids used had the following activities: L-tyrosine 7 or 10 $\mu$c/mg.; L-phenylalanine 7 or 10 $\mu$c/mg.; L-tryptophan 10 or 2 $\mu$c/mg.

*Experimental procedure.* Immediately after administration of the labelled amino acid the animal was placed, without food or water, in a metabolism apparatus similar to that devised by Lifson & Lorber (1954), allowing collection of respiratory $CO_2$, urine and faeces. After the appropriate time the animal was removed from the apparatus, anaesthetized with ether and rapidly dissected, and as much blood as possible removed from the aorta with a heparinized syringe. The liver, kidney, spleen, gut (including pancreas), heart, lung and brain were rapidly removed into a freezing mixture. One hind leg was then removed and the skin, muscle and bone were separated. It was assumed that the skin, muscle and bone of a hind leg were typical of the corresponding tissues of the whole animal, and calculations were based on the assumption that total muscle represented 50% of the body weight and that the weight of bone in the whole body was seven times that in the dissected leg. (These values were determined in a preliminary experiment.) In none of our experiments was significant activity found in the skin.

The treatment of the individual organs is exemplified by the following procedure for liver. The liver was washed, and weighed after removal of excess of water. A portion was then removed, weighed, cut up and dried in an oven at 100–120° for 12 hr. The dry residue was powdered and used for determination of total activity. The remaining portion of the liver was homogenized with 30 ml. of distilled water. The homogenate was placed for 5 min. in a boiling-water bath to coagulate the bulk of the protein. Trichloroacetic acid solution (30%, w/v) was added to a final concentration of 5% (or 10% for gut and muscle) and protein precipitation allowed to go to completion. The protein was spun down, washed with water and dried at 100–120° overnight. This material was used to determine 'protein-bound' activity. The combined supernatants from the protein precipitation were then shaken with 1 g. of charcoal (cf. Dalgliesh, 1955*a*). After time had been allowed for adsorption of aromatic substances the charcoal was filtered off, washed with water and dried and the activity determined. This value was taken to represent 'non-protein-bound aromatic' activity. It should be emphasized that this fractionation is crude. The 'protein-bound' activity may well include substances co-precipitated with the protein, and the 'non-protein-bound aromatic' activity may well include peptides or proteins not precipitable by heat and trichloroacetic acid.

The other organs were in general treated in the same way as the liver. The gut contents were thoroughly washed out before homogenization, and labelled amino acid which had not been absorbed was thereby removed. With gut it was particularly difficult to get an optically clear supernatant after the protein precipitation. For small organs, such as brain and lung, only total activity was determined. The bone was dried and powdered. The respiratory $CO_2$ was counted as $BaCO_3$.

In the shortest experiments no urine or faeces were usually produced. In the longer-term experiments the activity lost in urine and faeces was small, and these were therefore ignored. The total activity recovered in all organs and respiratory $CO_2$ was assessed, and values given in the paper are percentages of this recovery value. This was done to eliminate errors due to incomplete administration by stomach tube, or to incomplete absorption, the gut contents being thoroughly washed out and discarded.

*Activity determination.* Samples were counted at infinite thickness on 1, 2 or 5 sq.cm. planchets, a thin mica end-window Geiger tube and an automatic counter being used. Counts were continued to a standard error of $\pm 5\%$ for the lowest activities and $\pm 3\%$ for higher activities. For low-activity samples, determinations of background were made for each planchet in the appropriate position of the turntable of the automatic counter, before the planchet was made up with the labelled material. The following figures, obtained in the 3 hr. tyrosine experiment, show the orders of activity encountered. 5·6 $\mu$c of the amino acid were given. The total activity recovered in tissues and respiratory $CO_2$ was 86·9% of this amount. (All percentages in Figs. 1–4 relate to the amount recovered.) The counts in the 'protein bound' fraction of gut, liver, muscle and blood were respectively 284, 232, 22 and 105 counts/min./sq.cm. disk at infinite thickness. Under the same counting conditions a plastic standard equivalent to 1·02 $\mu$c/g. (as $BaCO_3$) gave 716 counts/min. Background was in the region of 7–10 counts/min.

## RESULTS

The experiments were largely dominated by the small amounts of labelled amino acids available. These dictated (*a*) that animal variation must be ignored, (*b*) that only single doses could be used, of not more than 6 $\mu$c for tyrosine or 2–4 $\mu$c for phenylalanine and tryptophan, for each time interval examined. The resultant low levels of activity make the accuracy in certain cases low, the more so as it was necessary to use relatively insensitive infinite-thickness counting techniques.

Moreover, many assumptions have had to be made. Thus we have assumed that administration of the amino acid under our experimental conditions approximates to the conditions obtaining during normal physiological intake. As the amount of amino acid administered did not exceed 1 mg. it is unlikely that an appreciable amino acid imbalance was induced. In order to eliminate difficulties due to incomplete administration or absorption, the activities found in the various tissues are expressed as percentages of the total activity recovered. Moreover, we have had to assume that different tissues, e.g. muscle, gut and liver, are each homogeneous. It is thus obviously unjustifiable to draw conclusions of more than a qualitative nature. But as experiments were carried out under comparable conditions we feel that with those organs containing an appreciable proportion of the administered activity the results are sufficiently accurate to permit comparisons of the metabolism of the three amino acids investigated.

Some indication of the relative overall rates of degradation of the three amino-acids is obtained by comparing the rates of appearance of activity in the respiratory $CO_2$, as shown in Fig. 1. The rate

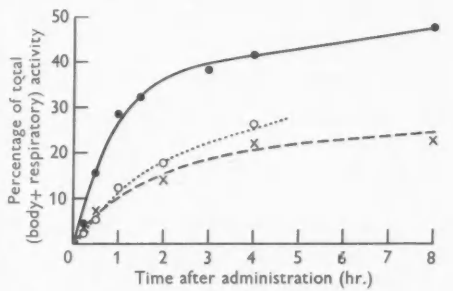


Fig. 1. Proportion of total absorbed activity appearing in the respiratory $CO_2$. ●, Tyrosine; ×, phenylalanine; ○, tryptophan. Each point derived from a different single animal.

of breakdown of tyrosine is rapid and is about double that of phenylalanine. Conversion of phenylalanine into tyrosine is the initial step of phenylalanine degradation, and this hydroxylation reaction must therefore be rate-limiting. The overall rate of breakdown of tryptophan is similar to that of phenylalanine. In normal degradation of tryptophan the alanine side-chain is lost and $C_2$ of the indole ring is liberated as formate. It seems unlikely that these degradation products can alone account for the observed rate of appearance of activity in respiratory $CO_2$. Thus, 1 hr. after tryptophan has been given, more than half the activity is in the 'protein-bound' or 'non-protein-bound aromatic' fractions. It is difficult to say to what extent the 'protein-bound' fraction represents tryptophan (e.g. it probably also includes alanine from side-chain scission), and similarly the 'non-protein-bound aromatic' fraction may have undergone some degradation. But it is not unreasonable to assume that at this time about half the tryptophan molecules are still intact. Of the activity originally in the remaining molecules one-quarter has therefore appeared as $CO_2$ after 1 hr. We consider this to be strong evidence that a ready route for degradation of the aromatic ring must exist.

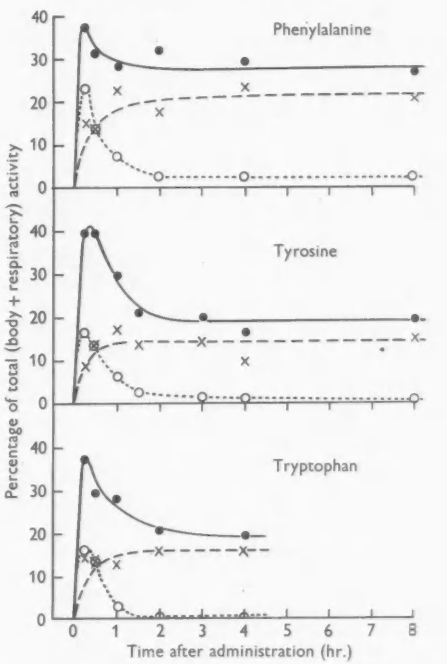


Fig. 2. Proportion of total absorbed activity appearing in muscle. ●, Total activity; ×, 'protein-bound' activity; ○, 'non-protein-bound aromatic' activity. Each point derived from a different single animal.

The organs containing much the largest proportions of the activity were muscle (Fig. 2), gut (Fig. 3) and liver (Fig. 4).

The results obtained with muscle in the tyrosine experiments are striking. The total activity rises to a high value and then falls, while protein activity rises more slowly. Less than one-quarter of the activity in muscle at 15 min. is protein-bound. Of

the non-protein-bound activity almost half (corresponding to about 15% of the total body activity) is present as non-aromatic substances. Obviously breakdown of the aromatic ring has been exceedingly rapid to allow such a large proportion of non-aromatic material to be present in the muscle after so short a time.

Similar changes were found in muscle in the phenylalanine experiments. There seems to be a higher proportion of the administered activity in muscle protein than with tyrosine, but this may represent both phenylalanine and derived tyrosine.

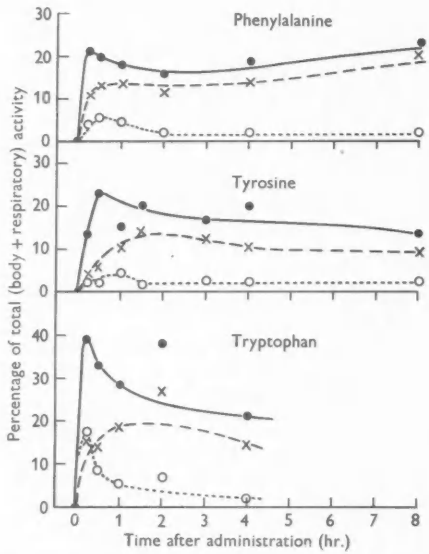


Fig. 3. Proportion of total absorbed activity appearing in gut. ●, Total activity; ×, 'protein-bound' activity; ○, 'non-protein-bound aromatic' activity. Each point derived from a different single animal.

With gut (Fig. 3) and liver (Fig. 4) the results with the three amino acids are again similar. Free amino acid (as represented by 'non-protein-bound aromatic' activity) is low in all except the shortest time intervals examined; this agrees with the findings of Tallan, Moore & Stein (1954), who have shown that in the cat free aromatic amino acids are present in the tissues in only small amounts. The relatively large proportion of the total body activity appearing in gut supports the existing evidence on the high-metabolic activity of the gut wall (see Fisher, 1954, for references; and Winnick, Friedberg & Greenberg, 1948, for similar evidence with DL-[$\beta$-$^{14}$C]tyrosine). It will be seen that a larger proportion of activity appears in the gut with tryptophan than with phenylalanine or tyrosine. We consider that this, taken with the evidence from Fig. 5 discussed below, indicates that the transfer process through the gut wall is

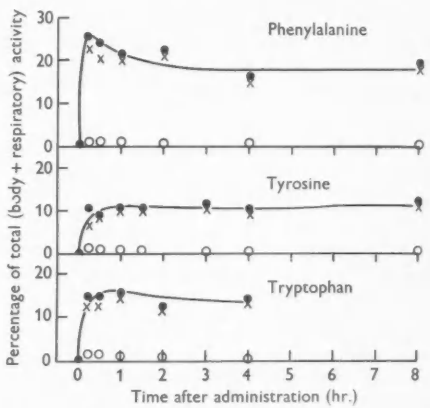


Fig. 4. Proportion of total absorbed activity appearing in liver. ●, Total activity; ×, 'protein-bound' activity; ○, 'non-protein-bound aromatic' activity. Each point derived from a different single animal.

The high values in the 'protein-bound' fraction after even short periods suggest that some of the activity may be coprecipitated with the protein without necessarily being in peptide combination. The general picture with tryptophan resembles that with phenylalanine. With all three amino acids there is a high initial non-protein-bound activity in the muscle which rapidly decreases, while protein incorporation more slowly increases. This situation is analogous to that found with glycine in rabbit muscle by Henriques, Henriques & Neuberger (1955), who have discussed its significance.

slower with tryptophan. In both gut and liver even at short time intervals the greater part of the total activity is accounted for by the 'protein-bound' fraction. It may be that in the early stages the 'protein-bound' fraction contains activity that is coprecipitated with protein rather than true protein, but it should be emphasized that the curves in Figs. 2–4 represent the proportion of total activity in the particular tissue rather than the absolute amount of labelled amino acid present. The scatter of the experimental points in Figs. 2–4 is probably to a large extent due to animal variation, each point representing only a single animal.

The remaining organs individually accounted for only a small proportion of the body content of activity. Surprisingly in all experiments bone was one of the more active organs (up to 4% of the total body activity), and this presumably reflects the metabolic activity of the marrow. Spleen, brain, heart and lung usually contained 0·5–2·0% each, and kidney somewhat more. The blood activity was particularly interesting in the experiments with tyrosine. At 15 min. the 'non-protein-bound

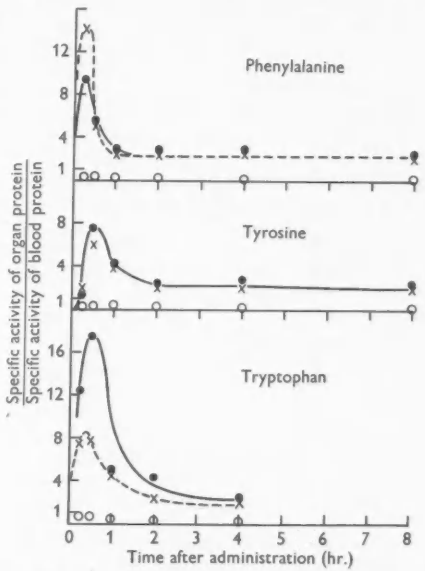


Fig. 5. Ratios of specific activities of 'protein-bound' fractions of muscle, gut and liver to specific activity of 'protein-bound' fraction of blood. ●, Gut; ×, liver; ○, muscle.

aromatic' fraction of blood contained 16·5% of the total body activity, but this had dropped to 1·2% at 30 min. The charcoal carrying the 15 min. 'non-protein-bound aromatic' fraction was eluted with aqueous phenol (cf. Dalgliesh, 1955*a*), the eluate was concentrated, and run on a paper chromatogram with butanol–acetic acid–water (4:1:5, by vol.; Partridge, 1946) as solvent, and a radioautograph made of the resulting chromatogram. Three radioactive substances were present of $R_F$ values 0·35 (corresponding to authentic tyrosine), 0·76 and 0·86. The spot of $R_F$ 0·76 was weak, but that of $R_F$ 0·86 was appreciably more intense than the tyrosine spot. We were unable to identify either of the fast-running substances with the limited amounts of material available, but they were probably not *p*-hydroxyphenyl-pyruvic or -acetic acids. Similar high activity at 15 min. was not found in the experiments with phenylalanine or tryptophan.

In Fig. 5 the specific activities of the proteins of liver, gut and muscle are compared with the activities of blood protein at the same times. It will be seen that the results for the three amino acids are generally similar. The liver and gut protein were more active than blood protein at all time intervals examined, but the ratio decreased rapidly in the early stages. Muscle protein was at all times less active than blood protein. The unusually high ratio of gut to blood protein at 15 and 30 min. in the tryptophan experiments, together with the evidence from Fig. 3, suggests that tryptophan remains longer in the gut wall than does either phenylalanine or tyrosine, i.e. the transfer of tryptophan through the gut wall may be a slower process.

## DISCUSSION

So far as we are aware no previous study of the overall metabolism of uniformly $^{14}$C-labelled aromatic amino acids has been made. By the use of uniformly, rather than specifically, labelled amino acids, it should be possible roughly to estimate overall metabolic rates. The limits of accuracy imposed by available materials and apparatus in the present work do not justify quantitative deductions; however, as experiments were carried out under similar conditions, it is thought that qualitative comparisons are valid.

Only small proportions of the phenylalanine and tyrosine ingested by a normal mammal are excreted as aromatic substances. The benzoic acid moiety of hippuric acid and the phenylacetic acid moiety of phenylacetylglutamine are probably derived at least partly from dietary phenylalanine either by direct conversion in body tissues (Schreier, Altman & Hempelman, 1954) or by the action of the bacterial flora of the gut. (The remainder may come from other aromatic substances in the vegetable matter in the diet.) Small amounts of aromatic precursors or degradation products of adrenaline, melanin and the thyroid hormones may also be present, together with traces of phenol and other products of baterial action. But the greater part of the normal intake of phenylalanine and tyrosine is converted into non-aromatic substances. This is easily explained by enzymic experiments (e.g. Knox & Le May-Knox, 1951; see also the detailed review by Dalgliesh, 1955*b*) which show that phenylalanine is converted into tyrosine, which is then degraded to acetoacetic and fumaric acids, both substances of high-metabolic reactivity. The great potential capacity of the body for tyrosine breakdown is well shown by the

experiments of Abderhalden (1912), who himself took 150 g. of tyrosine without causing abnormal excretion of aromatic metabolites.

The case of tryptophan is quite different, as can be seen by considering the known degradative pathways. A detailed discussion of the elucidation of these pathways has been given elsewhere (Dalgliesh, 1955*b*) and can be consulted for further references.

The major pathway of tryptophan degradation is considered to be that leading to nicotinic acid and its derivatives. This pathway is summarized in Fig. 6. In normal circumstances there is a small excretion of unchanged tryptophan and of nicotinic acid derivatives, but none of the other substances shown in Fig. 6 is excreted in appreciable amount. Presumably, therefore, at least one of the substances shown in Fig. 6 must be degraded to non-aromatic compounds. If nicotinic acid or nicotinamide is administered to any of a wide variety of species there is very little breakdown of the aromatic ring; instead, conjugation reactions of various types take place (cf. reviews: Kodicek, 1951; Dalgliesh, 1955*b*). The same applies to quinolinic acid. It is therefore reasonable to infer that the pyridine compounds are not the normal precursors of non-aromatic substances. Moreover, the discrepancy probably cannot be accounted for by incorporation into pyridine nucleotides, as in the steady-state neosynthesis of pyridine nucleotide should be balanced by an equivalent breakdown of preformed material. There is a possibility that breakdown of pyridine nucleotide involves opening of the pyridine ring before scission into smaller fragments, so that aliphatic substances result from this portion of the molecule when degradation occurs. However, the known ready exchange of free nicotinamide with the nicotinamide moiety of the pyridine nucleotides (Zatman, Kaplan & Colowick, 1953) suggests that incorporation and release of nicotinamide normally occur by such an exchange process.

The normal metabolism of kynurenine has been discussed in an earlier paper (Dalgliesh & Tekman, 1954), in which it was shown that by far the greater part is converted into hydroxykynurenine. As formylkynurenine gives kynurenine very readily, its fate can be regarded as ultimately similar. The normal fate of 3-hydroxykynurenine is to be split by the enzyme kynureninase to give 3-hydroxyanthranilic acid. The side reactions of kynurenine and 3-hydroxykynurenine are unlikely to account for more than a small proportion of the ultimate degradation of tryptophan to aliphatic compounds. For example, if anthranilic acid is administered to an animal it is almost entirely excreted free or as conjugates (Charconnet-Harding, Dalgliesh & Neuberger, 1953; Williams, 1949) and very little ring scission occurs. Kynurenic acid appears to be metabolically almost inert. Xanthurenic acid is not normally excreted, and though there is evidence that it can be degraded *in vivo* (Weitzel, Buddecke, Strecker & Roester, 1954; Kotake, Kotake & Inouye, 1954) it seems unlikely that such degradation is a rapid process that could account for more than a small proportion of the tryptophan degraded. Many of these intermediates can be excreted on giving a large dose of tryptophan. But this is probably largely due to adaptive increase in tryptophan peroxidase–oxidase, which converts tryptophan into formylkynurenine, without similar increase in the amounts of subsequent

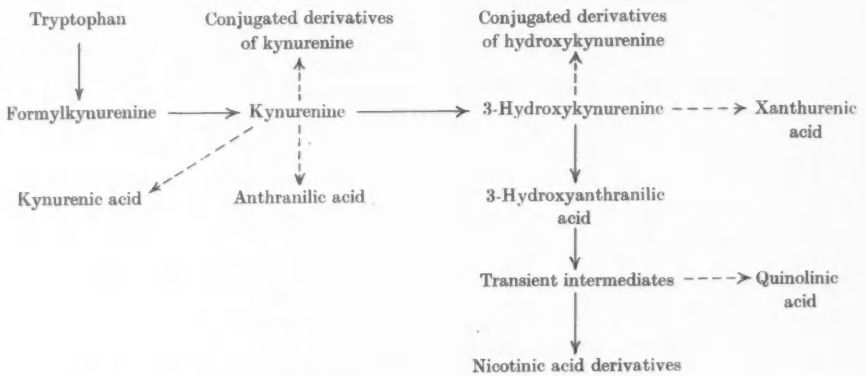


Fig. 6. Generally accepted major pathway for tryptophan degradation via nicotinic acid. Interrupted arrows represent side-reactions.

enzymes (cf. Dalgliesh & Tekman, 1954, for discussion). The amounts of tryptophan causing such abnormal excretion of intermediates are of the order of 100 or more times the 'physiological' amounts used in the present experiments.

The only remaining substance in the 'nicotinic acid pathway' of tryptophan metabolism is 3-hydroxyanthranilic acid. This is indeed a potential aliphatic precursor and is considered in more detail below.

A second pathway of tryptophan degradation is by way of 5-hydroxytryptophan, 5-hydroxytryptamine (enteramine, serotonin) and 5-hydroxyindolylacetic acid (for collected references see Dalgliesh, 1955*b*). Of these substances 5-hydroxytryptophan appears to be completely converted into 5-hydroxytryptamine. Very small amounts of hydroxytryptamine and derivatives are excreted (Bumpus & Page, 1955) and the major part is converted into hydroxyindolylacetic acid, which appears to be largely excreted as such (Erspamer, 1955; Page, Corcoran, Udenfriend, Szoedsma & Weissbach, 1955). Quantitative evidence (Erspamer, 1955) suggests that this pathway can account for only a small amount of the total tryptophan degraded (10–15 mg./day in man, compared with an intake of the order of 500 mg.).

There remain various routes for degradation of tryptophan by the bacterial flora of the gut. These are summarized in Fig. 7. The metabolites shown in

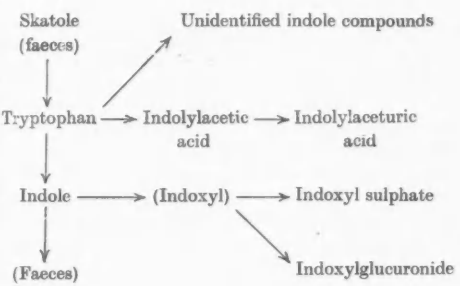


Fig. 7. Degradation of tryptophan by the bacterial flora of the gut.

Fig. 7 themselves account for a small amount of ingested tryptophan, but none appears to be a likely precursor of non-aromatic substances. Thus, administration of indolylacetic acid to an animal results in almost quantitative excretion of the free acid and its glycine conjugate, indolylaceturic acid (Ewins & Laidlaw, 1913; Schayer, Wu, Smiley & Kobayashi, 1954).

The experimental results described in this paper show that the rate of degradation of tryptophan is comparable with that of phenylalanine. It seems to be considerably greater than can be accounted for by our present knowledge of tryptophan metabolism. Various possibilities exist. First, there may be an unknown pathway or pathways of tryptophan degradation. The 5-hydroxytryptamine pathway is of remarkably recent discovery in spite of its physiological importance, and the existence of unknown pathways is by no means excluded. However, we consider it unlikely that a pathway accounting for a large part of the tryptophan normally degraded still remains undiscovered. Secondly, there may be a slow 'leakage' to non-aromatic compounds from several aromatic intermediates in the various tryptophan-degradative pathways. Although such 'leakage' may well contribute to tryptophan degradation we consider that it is unlikely to account for the comparatively rapid appearance of radioactivity in the respiratory $CO_2$ observed in the present experiments. Thirdly, an intermediate already known may be degraded by more than one route. There are two possible intermediates: the pyridine nucleotides—which we have discussed above—and 3-hydroxyanthranilic acid.

After giving the comparatively large amount of 600 mg. of 3-hydroxyanthranilic acid to a rabbit, Bray, Thorpe & Wood (1951) could account for only 13% of the dose as aromatic substances. It is therefore evident that ready breakdown of the aromatic ring occurs. In the intact rat 3-hydroxyanthranilic acid is converted into quinolinic acid to the extent of 8–15% as well as to appreciably smaller amounts of nicotinic acid and derivatives (Henderson, 1949). On the other hand, in rat-liver homogenates conversion of 3-hydroxyanthranilic acid into quinolinic acid is almost quantitative (70–100%; Bokman & Schweigert, 1950). Some transformation must therefore occur in the intact animal which does not occur in liver homogenates, presumably owing to inactivation of the appropriate enzyme. It seems to have been assumed that this other reaction is the formation of nicotinic acid, but the comparatively small amount of nicotinic acid formed when hydroxyanthranilic acid is given to an intact animal makes this unlikely, since it is known (as mentioned above) that most of the nicotinic acid formed is excreted as free or conjugated pyridine derivatives. It therefore seems likely that there is another, and major, pathway for hydroxyanthranilic acid degradation in the intact animal that allows formation of non-aromatic substances. Some conversion of hydroxyanthranilic acid into a red product can occur in certain liver preparations (Viollier & Süllmann, 1950), but this is probably an artifact, and may well be due to oxidation of hydroxyanthranilic acid

to phenoxazine derivatives analogous to those arising from hydroxykynurenine in insect eye-pigment formation (e.g. Butenandt, Schiedt & Biekert, 1954).

It is now clear, particularly from the enzymic work of Wiss (1954, 1955), that normal metabolism of 3-hydroxyanthranilic acid involves ring scission between carbons 3 and 4 to give an acroleinfumaric acid derivative (I, Fig. 8). It is also clear (for collected references see Dalgliesh, 1955*b*) that quinolinic acid formation from hydroxyanthranilic acid is a side reaction occurring by non-enzymic ring closure of an intermediate in nicotinic acid formation. quinolinic acid if $\beta$-decarboxylation of the imino-acid (II) occurred, may favour the carboxyl analogues of (IV) and (V). It is possible to envisage several schemes for conversion of compounds such as (IV) and (V) into substances likely to have a high metabolic-turnover rate, but their investigation must await enzymic experiments. A pathway such as suggested here could explain the comparatively rapid degradation of tryptophan in the intact animal; and if the appropriate enzymes are more labile than, or more easily separated from, those bringing about ring opening of hydroxyanthranilic acid, the much greater proportion of

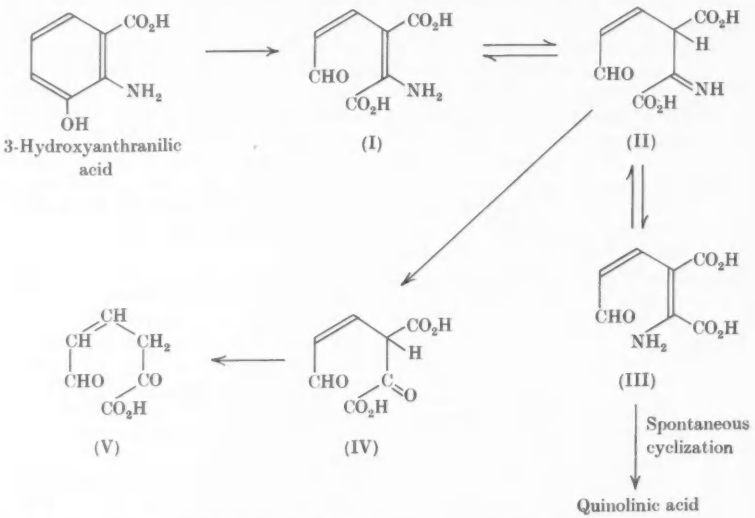


Fig. 8. Partly hypothetical scheme for the degradation of 3-hydroxyanthranilic acid.

The acroleinfumaric acid derivative (I) is most unlikely to be stable. Tautomerism to the imino acid derivative (II, Fig. 8) allows conversion into the acroleinmaleic acid derivative (III) which would almost certainly cyclize spontaneously to quinolinic acid. Nicotinic acid might be formed by decarboxylation of the $\alpha$-carboxyl group of (I), (II) or (III) before cyclization, and this is presumably an enzymic process. It is now suggested that either an appreciable part of hydroxyanthranilic acid metabolism can occur via the imino acid (II) to the keto acid (IV), in which the $\beta$-carboxyl group is doubly activated and will be readily lost to give the acroleinpyruvic acid (V), or the aldehyde group in (II) is first oxidized to a carboxyl group and similar reactions occur. The apparent absence of formation of picolinic acid, which might be expected to arise in an analogous way to

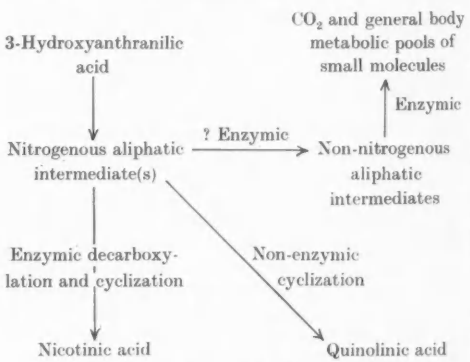


Fig. 9. Generalized scheme for breakdown of 3-hydroxyanthranilic acid.

hydroxyanthranilic acid converted into quinolinic acid in liver preparations compared with the intact animal can be readily explained. It is therefore proposed that it is via 3-hydroxyanthranilic acid that a larger part of tryptophan degraded *in vivo* reaches aliphatic metabolic pools, and that the metabolism of 3-hydroxyanthranilic acid can be summarized as shown in Fig. 9.

## SUMMARY

1. Adult rats starved for 16 hr. have been given solutions of uniformly $^{14}$C-labelled L-phenylalanine, L-tyrosine or L-tryptophan by stomach tube. The amount given (<1 mg.) was considered small enough to represent an approximation to physiological conditions. The radioactivity in respired carbon dioxide and in different organs was determined after varying intervals.

2. The rate of liberation of the label in the respiratory carbon dioxide showed that all three amino acids can be rapidly converted into non-aromatic substances of high metabolic activity. The overall rates of degradation of phenylalanine and tryptophan were similar, and about one-half that of tyrosine. Hydroxylation to tyrosine is thus the rate-limiting step in normal phenylalanine metabolism.

3. The rate of appearance of activity in whole muscle, liver and gut, and in the 'protein-bound' and 'non-protein-bound aromatic' fractions of these organs, is reported. At all times examined the specific activity of liver and gut protein exceeded that of blood protein, but muscle protein was less active.

4. In the existing state of our knowledge of tryptophan metabolism no pathway is known which appears to account for the observed comparatively rapid degradation of tryptophan. A survey of the various possibilities suggests that, unless a major pathway of tryptophan metabolism remains undiscovered, degradation is most likely to occur via 3-hydroxyanthranilic acid which, it is suggested, gives rise not only to nicotinic and quinolinic acids but also to non-nitrogenous substances with a high metabolic-turnover rate. Initial stages in the formation of such substances are proposed.

We thank Dr J. E. S. Bradley for advice and assistance with the automatic counter.

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# Component Acids of Suinsh Blubber and Liver Fats

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(*Received* 29 *August* 1955)

Pathak & Agarwal (1952) have studied the component acids of the fats from different parts of a freshwater fish *Catla buchanani*. Fats from the body and the viscera of two other Indian freshwater fishes, *Labeo rohita* and *Cirrhina mrigala*, have been investigated by Pathak, Pande & Mathur (1954). The present investigation deals with the blubber and liver fats from an Indian freshwater mammal, a Ganges dolphin (*Platanista gangetica*; local name, suinsh) caught in the River Ganges near Banaras in May 1952. The mammal was a female about 4 ft. in length and weighing about 100 lb.

## EXPERIMENTAL

Portions of the blubber and of the liver of the mammal were cut up into small pieces and separately extracted several times with hot acetone. The acetone was removed and the residue was treated with light petroleum (b.p. 40–60°) at room temperature, and the lipids were extracted. The lipids were recovered from the solution and dried under vacuum, and kept in 10 vol. of acetone at 0° for several weeks to separate the phosphatides, but none separated.

The mixed fatty acids obtained in the usual manner were resolved into groups of simpler mixtures of acids differing in unsaturation by the lithium salt–acetone method (Tsujimoto, 1920; Tsujimoto & Kimura, 1923) followed by the lead salt–ethanol method (Hilditch, 1949). Each group of acids was separately converted into methyl esters in the usual manner (Bjarnason & Meara, 1944) and fractionated through an electrically heated and packed column (Pathak *et al.* 1954). Saponification equivalents and iodine values of each of the subfractions were determined and the composition was calculated from them (Hilditch, 1949). The mean unsaturation, expressed by the fractional number of the hydrogen atoms short of saturation (e.g. −2·0, mono-ethenoid), was determined as usual by interpolation or extrapolation from the respective ester fractions in each group. The compositions of the original fats were obtained from those of the separated groups of fatty acids.

## RESULTS

*Blubber fat.* The mixed fatty acids (247·0 g., iodine value 98·0) were resolved into three groups. The results are recorded in Table 1. The component acids of each group and of the whole fat are recorded in Table 2.

Table 1. *Fractions of suinsh-blubber fatty acids*

Oil (270·0 g.: saponification equivalent 280·8; iodine value 98·6) gave on saponification 253·0 g. of mixed fatty acids. The Reichert–Meissl and Reichert–Polensky values were 0·2 and 0·5 respectively.

| Fraction | Description | Wt. (g.) | Wt. (%) | Iodine value |
|---|---|---|---|---|
| A | Lead salt, ethanol-insoluble | 46·7 | 18·9 | 12·2 |
| B | Lithium salt, acetone-insoluble, but lead salt, ethanol-soluble | 164·0 | 66·4 | 101·5 |
| C | Lithium salt, acetone soluble | 36·3 | 14·7 | 158·6 |

Table 2. *Component acids of suinsh-blubber fat*

| | Component acids in groups as percentage of total | | | Component acids in whole fat (excluding unsaponifiable fraction) | | |
|---|---|---|---|---|---|---|
| Acid | A (18·9 %) | B (66·4 %) | C (14·7 %) | % (w/w) | % (mol.) | Mean unsaturation (H atoms/mol.) |
| Lauric | — | 0·3 | — | 0·3 | 0·4 | — |
| Myristic | 1·4 | 2·6 | — | 4·0 | 4·7 | — |
| Palmitic | 11·2 | 2·8 | — | 14·1 | 14·7 | — |
| Stearic | 4·3 | 3·1 | — | 7·3 | 7·0 | — |
| Unsaturated fractions | | | | | | |
| $C_{14}$ | Trace | 3·1 | 1·1 | 4·2 | 5·0 | −2·0 |
| $C_{16}$ | 0·5 | 18·7 | 2·9 | 22·1 | 23·4 | −2·1 |
| $C_{18}$ | 1·5 | 34·0 | 3·9 | 39·5 | 37·6 | −2·7 |
| $C_{20}$ | — | 1·8 | 3·6 | 5·4 | 4·7 | −5·7 |
| $C_{22}$ | — | — | 3·1 | 3·1 | 2·5 | −8·5(?) |
| Unsaponifiable fraction | Trace | Trace | 0·1 | — | — | — |

*Liver fat.* The quantity of the liver fat available (30·0 g.) was too small for resolution into simpler mixtures of acids by the lithium-salt or the lead-salt method, hence the esters of the mixed acids were directly fractionated in the same way as pahuna-liver fat (Pathak & Agarwal, 1952). The calculated composition of the fat is recorded in Table 3.

Table 3. *Component acids of suinsh-liver fat*

A sample (30·0 g.) of the liver fat (iodine value 95·2; saponification equivalent 284·5) gave 27·3 g. of mixed fatty acids.

| Acid | % (w/w) | % (mol.) |
|---|---|---|
| Myristic | 7·7 | 9·1 |
| Palmitic | 7·4 | 7·8 |
| Stearic | 4·5 | 4·2 |
| Unsaturated fractions | | |
| $C_{14}$ | 5·8 | 6·9 |
| $C_{16}$ | 13·3 | 14·0 |
| $C_{18}$ | 30·3 | 30·5 |
| $C_{20}$ | 26·1 | 24·1 |
| $C_{22}$ | 4·0 | 3·4 |
| Unsaponifiable fraction | 0·9 | — |

## DISCUSSION

The mammal *Platanista gangetica* belongs to the zoological suborder Dontoceti (Delphinidae), to which also belong sperm whales, dolphins and porpoises. Few totally aquatic mammals inhabit fresh waters exclusively, and this is one of the rare species that has left the sea and inhabited the rivers. A few other species belonging to this family are found in the rivers of South America.

*Blubber fat.* Negligible values for Reichert–Meissl (0·2) and –Polenske (0·5) estimations indicate the absence of lower acids in suinsh-blubber fat. The species is rare, so that comparative data are not available. The composition of the fat lies between that of typical marine-mammal fat and that of the typical freshwater fish. For comparison the fatty acid compositions of fats of some marine mammals and some freshwater fishes are given in Table 4.

The fat largely resembles the sperm-whale fat; the important difference is its lower content of unsaturated $C_{20}$ acids, which is made up by a higher content of palmitic and stearic acids. The general unsaturation is higher than that of the sperm-whale fat. There is a close resemblance between the two in their content of other acids.

The saturated acids of suinsh fat differ from those of porpoise fat chiefly in the absence of *iso*-valeric acid, a lower content of lauric and myristic acids, and a higher content of palmitic and stearic acids. In the unsaturated portion of suinsh fat the chief feature is the much higher proportion of unsaturated $C_{18}$ acids than that in porpoise fat. Incidentally, a higher content of unsaturated $C_{18}$ acids is characteristic of fats of freshwater fishes. The contents of unsaturated $C_{16}$, $C_{20}$, and $C_{22}$ acids are slightly higher in porpoise fats.

Suinsh fat shows a somewhat closer resemblance to dolphin fat than to porpoise fat. The saturated portion differs from both porpoise and dolphin fat in a similar manner. In the unsaturated portion the similarity to dolphin fat is closer, both in the percentages of acids of different carbon content and in the degrees of their unsaturation, except for the much higher content of unsaturated $C_{20}$ acids in dolphin fat (18·6 %) than in suinsh fat (5·4 %). The absence of *iso*valeric acid and reduced proportions of lower saturated acids in the blubber fat from an animal of the same family as porpoise and dolphin is notable.

Table 4. *Component acids of blubbers of some marine mammals and body fats of freshwater fishes*

Amounts of the acids are given in % (w/w). Figures in parentheses show the mean unsaturation.

| | Marine mammals | | | | Freshwater fish body fat | | |
|---|---|---|---|---|---|---|---|
| Acids | Suinsh | Sperm whale* | Porpoise† | Dolphin† | Nain‡ | Rohu‡ | Bhakur§ |
| *iso*Valeric | — | — | 13·6 | 3·2 | — | — | — |
| Lauric | 0·3 | 1·0 | 3·5 | 1·0 | — | — | — |
| Myristic | 4·0 | 5·0 | 12·1 | 7·2 | 1·9 | 3·4 | 2·9 |
| Palmitic | 14·1 | 6·5 | 4·7 | 8·6 | 21·4 | 21·2 | 29·0 |
| Stearic | 7·3 | — | — | 0·8 | 3·1 | 11·5 | 6·5 |
| Arachidic | — | — | — | — | — | 0·5 | — |
| Unsaturated fractions | | | | | | | |
| $C_{14}$ | 4·2 (−2·0) | 4·0 (−2·0) | 4·7 (−2·0) | 4·7 (−2·0) | 3·7 (−2·0) | 3·7 (−2·0) | 0·2 (−2·0) |
| $C_{16}$ | 22·1 (−2·1) | 26·5 (−2·0) | 27·2 (−2·0) | 25·9 (−2·0) | 32·6 (−2·2) | 8·1 (−2·2) | 25·3 (−2·7) |
| $C_{18}$ | 39·5 (−2·7) | 37·0 (−2·0) | 16·7 (−2·8) | 24·1 (−3·3) | 29·5 (−3·6) | 32·2 (−3·3) | 17·9 (−2·5) |
| $C_{20}$ | 5·4 (−5·7) | 19·0 (−2·5) | 10·5 (−4·8) | 18·6 (−6·5) | 5·0 (−4·9) | 12·4 (−5·2) | 7·5 (−5·7) |
| $C_{22}$ | 3·1 (−8·5) | 1·0 (−4·0) | 7·0 (−4·9) | 5·9 (−7·6) | 2·8 (−6·0) | 6·7 (−6·9) | 10·1 (−9·0) |
| $C_{24}$ | — | — | — | — | — | 0·3 (−8·0) | 0·6 (−11·0?) |

* Hilditch & Lovern (1928, 1929).
† Lovern (1934).
‡ Pathak, Pande & Mathur (1954).
§ Pathak & Agarwal (1952).

As suinsh inhabits fresh water, it is natural that its fat has some similarities to fat of freshwater origin. This becomes evident by comparing its composition with that of nain-body fat and of bhakur-body fat (Table 4). It is observed that the palmitic acid content of suinsh fat is lower than that of the fats of freshwater fishes, but higher than that of the fats of the marine mammals. The situation is similar with the $C_{16}$ unsaturated acids, except in rohu fat, where the $C_{16}$ unsaturated acid content is rather low.

richer in lower-molecular-weight saturated acids, as well as in unsaturated acids, than the seal-liver fat. Suinsh- and porpoise-liver fats resemble each other closely in their content of palmitic, stearic and unsaturated $C_{20}$ acids, but differ widely in their content of myristic and unsaturated $C_{14}$, $C_{16}$, $C_{18}$ and $C_{22}$ acids.

The content of unsaturated $C_{18}$ acids in pahuna-liver fat is similar to that in suinsh-liver fat. With all the other acids there is the similarity generally found with lower-molecular-weight acids predomi-

Table 5. *Component acids of the liver fats of some aquatic animal fats*

Amounts of the acids are given in % (w/w). Figures in parentheses show the mean unsaturation.

| | Marine mammals | | | Freshwater fishes | |
|---|---|---|---|---|---|
| Acid | Suinsh | Common seal* | Porpoise† | Pahuna‡ | Bhakur‡ |
| Myristic | 7·8 | 0·2 | — | 1·5 | 0·6 |
| Palmitic | 7·5 | 11·4 | 7·6 | 14·2 | 19·0 |
| Stearic | 4·5 | 7·8 | 5·5 | 4·0 | 6·4 |
| Arachidic | — | 0·1 | — | — | 3·1 |
| Unsaturated fractions | | | | | |
| $C_{14}$ | 5·8 (−2·0) | — | — | 0·4 (−2·0) | Trace |
| $C_{16}$ | 13·4 (−2·0) | 8·6 (−2·0) | 6·1 (−2·0) | 8·4 (−2·0) | 7·0 (−2·0) |
| $C_{18}$ | 30·6 (−2·6) | 27·9 (−2·4) | 42·5 (−2·8) | 32·6 (−2·4) | 25·4 (−2·7) |
| $C_{20}$ | 26·3 (−7·8) | 23·7 (−6·2) | 27·3 (−5·4) | 19·8 (−5·3) | 26·5 (−4·8) |
| $C_{22}$ | 4·1 (−10·0?) | 20·3 (−11·0?) | 11·0 (−6·5) | 19·1 (−8·0) | 9·4 (−6·5) |
| $C_{24}$ | — | — | — | — | 2·6 (−8·0) |

* Hilditch & Pathak (1949). † Lovern (1934). ‡ Pathak & Agarwal (1952).

The comparatively greater proportion of unsaturated $C_{18}$ acids (nearly 40 %) in fat from suinsh may probably be explained by its adoption of a freshwater habitat, since increased proportions of unsaturated $C_{16}$ and $C_{18}$ acids and smaller proportions of unsaturated $C_{20}$ and $C_{22}$ acids are characteristic of freshwater animal fats; this is evident from the compositions of nain- and bhakur-fish fats given in Table 4. Suinsh fat resembles crocodile fat (Pathak & Pande, 1955) closely, except for its higher content of unsaturated $C_{16}$ acids, although the total content of $C_{16}$ acids remains practically the same.

When suinsh-body fat and suinsh-liver fat are compared it is noticed that the relationship between them is very much the same as that described by Pathak & Agarwal (1952) for bhakur fats. The body fat has a higher content and the liver has a lower content of saturated acids, especially of palmitic acid.

A general survey of these two fats confirms the view expressed by Lovern (1942) that the chemical composition of the fat is greatly influenced by the biological origin and the environment.

*Suinsh-liver fat.* Suinsh-liver fat resembles common seal-liver fat (Hilditch & Pathak, 1949) in the total saturated-acid content (Table 5). In the unsaturated-acid portion the contents of $C_{18}$ and $C_{20}$ acids are similar in both animals. Suinsh fat is nating in suinsh fat. The content of unsaturated $C_{20}$ acids is more in suinsh fat. It resembles bhakur-liver fat in the content of $C_{20}$ acids, but in other acids suinsh fat is generally richer in lower-molecular-weight acids and poorer in higher-molecular-weight acids.

## SUMMARY

1. The compositions of blubber and liver fats of suinsh (*Platanista gangetica*) have been studied. Preliminary separations of the fatty acids into groups differing in their content of unsaturated acids were made with lithium and lead salts in acetone and ethanol respectively, and their compositions were studied by the ester-fractionation procedure.

2. Suinsh is a mammal which has left its original habitat, the sea, and has inhabited fresh water. Its blubber fat resembles dolphin and porpoise fats in composition to some extent, but also resembles a typical fat from freshwater fishes. This supports the present views on the effect of the biological origin and of the environment on the composition of the fat. The suinsh-blubber fat does not contain *iso*valeric acid, which is characteristic of dolphin and porpoise fat, and in this respect it is unique.

3. The liver fat shows the usual significant differences in composition from the body fat.

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# Comparative Studies of 'Bile Salts'

## 9. THE ISOLATION AND CHEMISTRY OF HYOCHOLIC ACID*

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(*Received* 14 *June* 1955)

The bile of the domestic pig is known to contain 3α:6α-dihydroxycholanic acid (hyodeoxycholic acid; Windaus & Bohne, 1923), 3α:7α-dihydroxycholanic acid (chenodeoxycholic acid; Ido & Sakurai, 1939), 3β:6α-dihydroxycholanic acid (Kimura, 1937) and 3α-hydroxy-6-oxo*allo*cholanic acid (or 3α-hydroxy-6-oxocholanic acid; Fernholz, 1935), all probably in conjugated form. Shimizu (1953) isolated 3α-hydroxycholanic (lithocholic) acid from the bile, and Trickey (1950) suggested that an acid of formula $C_{27}H_{44}O_6$ might be present. Pig bile acids are largely conjugated with glycine, but taurine derivatives also occur (Haslewood & Sjövall, 1954). The glycine conjugates were examined chromatographically by Haslewood & Sjövall (1954), who detected a substance which on paper ran rather more slowly than glycocholic acid. The parent trihydroxy acid, $C_{24}H_{40}O_5$, probably corresponding to this compound, was later isolated (Haslewood, 1954*a*) and named hyocholic acid. The present report describes the isolation and properties of this substance, and gives an account of experiments which have almost entirely elucidated its chemical constitution. In this work unexpected difficulties were encountered.

## RESULTS

Pig bile salts were hydrolysed by recognized methods in an autoclave or, by the use of ethylene glycol, at atmospheric pressure. The ethylated crude bile acids were separated on alumina columns, and fractions were examined by paper chromatography as previously described (Haslewood, 1954*b*). Esters of hyodeoxycholic and chenodeoxycholic acids were easily recognized. There were also spots corresponding to at least two unidentified substances. One of these remains unidentified, but is not ethyl 3β:6α-dihydroxycholanate or ethyl 3α-hydroxy-6-oxo*allo*cholanate; the other was shown to be ethyl hyocholate. A simple chromatographic process then permitted the isolation in substance of hyocholic acid, which could also be obtained, without chromatography, from crude 'hyodeoxycholic acid', as usually prepared. Girard separation of the ethylated bile acids removed ethyl 3α-hydroxy-6-oxo*allo*cholanate almost quantitatively in a single operation; it did not remove ethyl hyocholate. Hyocholic acid was converted into a crystalline ethyl ester, diacetyl ethyl ester, triacetyl methyl ester, triacetyl ethyl ester and other derivatives, discussed below. Triacetyl methyl hyocholate and triacetyl ethyl hyocholate readily formed characteristic crystals, resembling those of cholesterol. The triacetyl ethyl ester was useful in the isolation of hyocholic acid from mixtures.

The most obvious property of hyocholic acid itself was its similarity to hyodeoxycholic acid, from which it could not easily be separated by ordinary crystallization. Purified hyocholic acid had m.p. 188–189° and $[\alpha]_D + 5{\cdot}5°$ in ethanol; the mixed m.p. with hyodeoxycholic acid (m.p. 197–198°, $[\alpha]_D + 7°$) was 184–196°. Other mixtures of hyodeoxycholic and hyocholic acids melted sharply; for example, a laboratory specimen of 'hyodeoxycholic acid' of m.p. 191–192° was shown to contain at least 20% of hyocholic acid. Much work may have been done in the past on 'hyodeoxycholic acid' contaminated in this way.

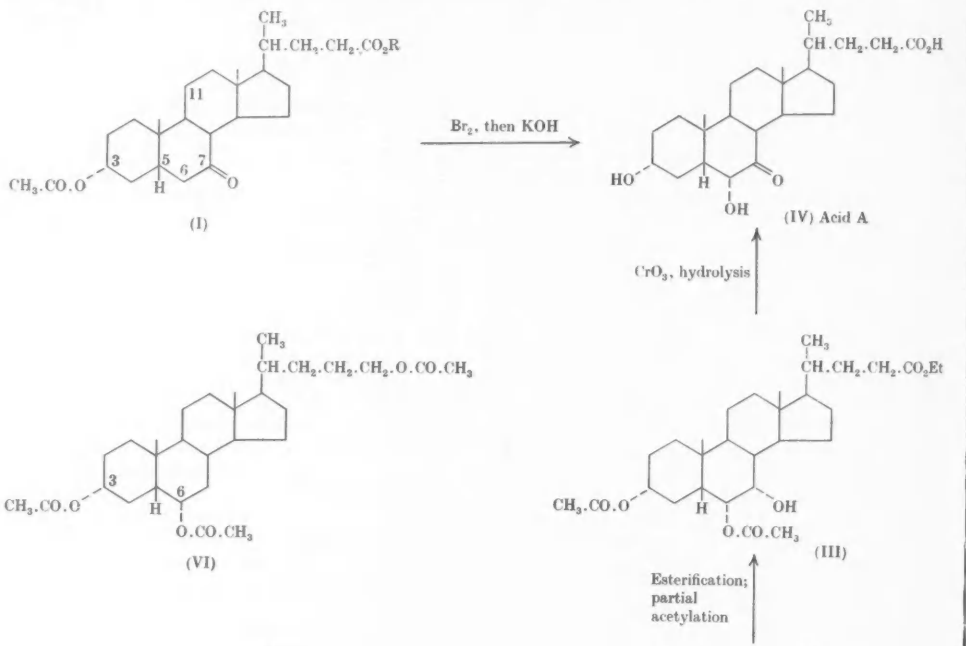
* Part 8: Haslewood & Sjövall (1954).

Hyocholic acid on chromic oxidation gave a (non-crystalline) product which with alkali gave a yellow colour. Windaus (1926) observed this behaviour with dehydrohyodeoxycholic (3:6-dioxocholanic) acid, and Hoehn, Linsk & Moffett (1946) similarly reported that 6-oxocholanic acid dissolved in aqueous sodium hydroxide with development of a yellow colour. This colour is presumably associated with enolization of the C-6 oxocholanic acids, and its appearance in the present case was regarded as evidence for the presence in hyocholic acid of a hydroxyl group at C-6 in a cholane nucleus.

Almost all known indubitably 'native' bile acids are 3α-hydroxy compounds, and hyocholic acid was thus provisionally formulated as a 3α:6:*x*-trihydroxycholanic acid.

Mild acetylation of ethyl hyocholate gave a diacetate; complete acetylation could be effected by the perchloric acid method or after prolonged heating. The diacetate was converted by chromic oxidation into a different substance of almost identical elementary composition; this substance was presumably a ketone (or aldehyde), but it did not form a Girard complex or a semicarbazone, and gave no recognizable product on Clemmensen or Kishner–Wolff reduction. Hence the third hydroxyl group in hyocholic acid was (*a*) primary or secondary, (*b*) difficult to acetylate, and (*c*) convertible into a very unreactive keto (or aldehyde) group.

An 11α-hydroxyl group would have properties (*a*) and (*c*), and the possibility of the existence of such a group was supported by optical-rotation measurements. The (presumed) 3α:6-dihydroxy-*x*-oxocholanic acid (A) derived from the unreactive



'ketone' decomposed at about 215° and had an $[M]_D$ (about +50°), which differed little from that of hyocholic acid (about +22°). These findings excluded position C-12 and supported C-11 for a hydroxyl group having the properties enumerated (Barton & Klyne, 1948; Haslewood & Wootton, 1951). No other position, as far as was then known, could be seriously considered and hyocholic acid was tentatively formulated as 3α:6α:11α-trihydroxycholanic acid (Haslewood, 1954*a*, 1955).

However, as evidence was secured, this possibility seemed more and more unlikely. The diacetate did not form a *p*-tosylate, and could not be satisfactorily dehydrated with $POCl_3$; the derived 'ketone' on catalytic hydrogenation was converted not into the expected 11β-hydroxy compound but largely into the original diacetate. Reduction of triacetyl ethyl hyocholate with $LiAlH_4$ gave, in good yield, a tetraol, presumably 3α:6:*x*:24-tetrahydroxycholane, which gave a triacetate and a tetraacetate. The triacetate, like diacetyl ethyl hyocholate, could be oxidized to a ketone (or aldehyde) unreactive towards semicarbazide and giving no recognizable product after treatment by the Kishner–Wolff method. It could not thus be converted into 3α:6α:24-triacetoxycholane (VI) prepared after $LiAlH_4$ reduction of ethyl hyodeoxycholate. In other respects, also, the $LiAlH_4$ product (tetraol) from triacetyl ethyl cholate behaved like the parent ester, in that its triacetate could not be satisfactorily dehydrated with $POCl_3$ and the derived 'ketone' regenerated the original triacetate almost quantitatively on reduction with $LiAlH_4$.

Finally, it was found that cold chromic acid oxidation of ethyl hyocholate gave, in substantial yield, an *acidic* product, which was subsequently converted by Kishner–Wolff reduction and hydrolysis into a substance having the properties of thilobilianic acid (V). This observation could by no means be reconciled with the 3α:6α:11α-trihydroxy formula, but indicated that two of the hydroxyl groups must occupy adjacent positions in ring II.

Among the new possibilities for a hyocholic acid formula was 3α:6α:7-trihydroxycholanic acid (II). When ethyl 3α-acetoxy-7-oxocholanate (I, R = Et) or the acid (I, R = H) was brominated and the product boiled with methanolic potassium hydroxide, there was isolated an acid, decomposing at about 215°, which was identical with the acid A mentioned above. This acid is probably 3α:6α-dihydroxy-7-oxocholanic acid (IV, see Discussion), and hyocholic acid is therefore 3α:6α:7-trihydroxycholanic acid (IV). Some evidence was obtained that hyocholic acid formed an acetonide, although no crystalline derivative could be made; acetonide formation would indicate that the hydroxyl groups at C-6 and C-7 are probably in the *cis* configuration, i.e. the hydroxyl group at C-7 is in the α-position.

## EXPERIMENTAL

*General.* Melting points were determined on a Kofler-block type of apparatus and are corrected. $Al_2O_3$ was Spence 'Type H' (Peter Spence, Widnes) except where otherwise stated. Light petroleum had b.p. 40–60°. 20% $CrO_3$ was a solution of about 20 g. of $CrO_3$ in a minimal vol. of water made, with mixing, to 100 ml. with acetic acid. Microanalyses (C, H) were by Drs Weiler and Strauss, Oxford.

*Esterification.* Process *A* consisted in leaving the dried acid (1 part) in methanol or ethanol (10 parts) containing 2% (v/v) $H_2SO_4$ for at least 16 hr., with occasional shaking, sufficient to cause complete solution and mixing. The mixture was then diluted with water and extracted 2–3 times with ether. The ether was washed with water, aqueous ammonia and water, dried ($Na_2SO_4$) and evaporated; the residue was 'esters'. Acidification of the aqueous ammonia washings gave 'unesterified acids'.

### *Hydrolysis of pig bile salts*

(*a*) *At ordinary pressure.* Bile salts (50 g., prepared as described by Haslewood & Wootton, 1950) were dissolved in a mixture of KOH (25 g.), water (120 ml.) and ethylene glycol (250 ml.). The solution was boiled for 3 hr., with loss of vapour, until the temp. rose to 140°. A reflux condenser was then fitted and the mixture simmered, gently to avoid frothing, for a further 10 hr. at 138–142°. The cooled product was diluted with water (1·5 l.) and acidified with 5N-HCl (excess). After 3 days, the liquors were decanted, and the washed residue, dried by evaporation with ethanol, was stirred with ethyl acetate. The filtered solution was evaporated and the residue dissolved in 50% (v/v) acetone–ethyl acetate. Crystals slowly formed, and after about 20 days at about 5° these were collected and washed with cold 50% (v/v) acetone–ethyl acetate. Yield, about 5 g., m.p. 185–188°. This material was later shown to be approximately 80% (w/w) hyodeoxycholic–hyocholic acids. The ethyl acetate liquors were evaporated and the residue (33 g.) ethylated by process *A*, giving 'ethyl esters' (32 g.) as a dark-brown gum.

(*b*) *At increased pressure.* Bile salts (40 g.) were dissolved in a 500 ml. beaker with KOH (24 g.), water (120 ml.) and ethanol (120 ml.). The beaker, covered with a clock glass, was placed in a 'pressure cooker' containing about 800 ml. of water, and the cooker was sealed and heated at 35–40 lb. pressure/in.$^2$ for 6 hr. When the cooled cooker was opened, it was found that part of the alkaline 'bile acid' solution had frothed out of the beaker. The frothed liquors were collected separately; acidification of these gave a solid precipitate (2·1 g.) which, after washing and drying, separated easily from ethyl acetate as crystals (0·5 g.) of m.p. 182–185°, subsequently shown to be approximately 80% (w/w) hyodeoxycholic–hyocholic acids. Acidification of the diluted contents of the beaker gave the 'bile acids'; these were collected, washed, evaporated to dryness with ethanol *in vacuo* and esterified by process *A*. Yield, 28 g. of 'ethyl esters'.

### *Separation of 'ethyl' esters and bile acids*

*Preliminary separation.* The 'esters' (8·7 g., prepared as under (*a*) above) were dissolved in benzene (about 20 ml.) and the solution was poured on a column (diam. 2·9 cm.) of

$Al_2O_3$ (100 g.) prepared in benzene. Fractions were eluted and each fraction was examined by paper chromatography with Bush's (1952) system *A* as previously described (Haslewood, 1954*b*). Results are shown in Table 1. Unknown X ran at rather more than half the rate of ethyl chenodeoxycholate and unknown Z rather more slowly than ethyl cholate. X has not yet been identified; it cannot be ethyl 3$\beta$:6$\alpha$-dihydroxycholanate or ethyl 3$\alpha$-hydroxy-6-oxo*allo*cholanate, for these were not detected on chromatograms under the conditions described above. Z corresponded in position to ethyl hyocholate. In some chromatograms there was an indication of an unknown Y, running at a rate between those of ethyl hyodeoxycholate and ethyl hyocholate.

The crystals in fraction II were isolated, recrystallized and shown (m.p. and mixed m.p.) to be largely ethyl 3$\alpha$-hydroxy-6-oxo*allo*cholanate (see below).

ether, which was then used to dissolve the precipitate. The ether solution was washed with water, aqueous $NH_3$ and water; acidification of the washings gave 'ketonic acids' (0·156 g.). The dried ($Na_2SO_4$) ether was evaporated to give 'ketonic esters' (1·07 g.) as a partly crystalline solid. Repetition (twice) of the above process on the 'non-ketones' (17·00 g.) gave results as follows: 1st repetition: 'non-ketones' (16·77 g.); 'ketones' (0·045 g.). 2nd repetition: 'non-ketones' (16·43 g.); 'ketones' (0·02 g.). Hence, allowing for working losses, removal of the ketonic material from pig bile esters is almost completed by one Girard extraction; this finding is in contrast to observations on coypu bile acids (Haslewood, 1954*b*).

The first 'ketonic acids' (above) appeared to be impure 3$\alpha$-hydroxy-6-oxo*allo*cholanic acid. Crystallization of the first 'ketonic esters' (1·07 g.) from ether gave a first crop (0·48 g., m.p. 135–136°). Evaporation of the filtrate left

Table 1. *Chromatography of ethylated pig 'bile acids'*

The 'bile acids' (8·7 g.) were fractionated on $Al_2O_3$ (100 g.). Each fraction was examined by paper chromatography as described in text. ECh = spot as ethyl chenodeoxycholate; EHd = spot as ethyl hyodeoxycholate. For details of unknowns X, Y and Z see text.

| Fraction no. | Eluted by (solvent, ml.) | Wt. (g.) and appearance | Result of paper chromatography |
|---|---|---|---|
| I | Benzene, 320 | 1·75, oil | No spots |
| II | Benzene, 150 | 0·31, oil + crystals | No spots |
| III, IV | Benzene, 150 each | 0·30, gum | ECh + EHd + unknown X |
| V | 20% (v/v) Ether–benzene, 100 | 0·09, gum | |
| VI | As above | 0·16, gum | |
| VII | 50% (v/v) Ether–benzene, 100 | 0·23, gum | |
| VIII to X | Ether, 100 each | 0·90, gum | |
| XI | Ether, 200 | 0·83, gum | |
| XII, XIII | Ether, 200 each | 0·76, gum | ECh + EHd only |
| XIV | Ether, 400 | 0·47, gum | |
| XV | Ether, 400 | 0·31, crystals | |
| XVI | Ether, 600 | 0·30, gum | |
| XVII | Ether, 600 | 0·18, gum | ECh + EHd + ? unknown Y |
| XVIII | Ether, 600 | 0·04, gum | EHd + ? unknown Y |
| XIX | Ether, 600 | 0·03, gum | |
| XX, XXI | Acetone, 200 each | 0·52, gum | EHd + ? unknown Y + unknown Z |
| XXII, XXIII | Ethanol, 200 each | 0·86, gum | Unknown Z only |

Total eluted = 8·04 g.

Fraction XV was crystallized from 25% (v/v) ether–light petroleum and thus gave short white needles of ethyl hyodeoxycholate (Haslewood, 1954*b*), m.p. 111–114°, which showed only one spot on paper chromatography. Hydrolysis of this yielded pure hyodeoxycholic acid, as glistening needles, m.p. 197–198°; $[\alpha]_D^{23} + 6{\cdot}9 \pm 0{\cdot}5°$ in ethanol (*c*, 2·1).

*Girard separation.* Pig bile acid 'esters' (18·7 g., prepared as under (*b*) above and carefully dried *in vacuo* over $H_2SO_4$) were boiled for 1 hr. under reflux with dry ethanol (100 ml.), acetic acid (10 ml.) and Girard's reagent 'T' (5 g.). The cooled mixture was diluted with ice water (3–4 vol.) and adjusted to pH about 7, with bromothymol blue as external indicator, by addition of 5N- and then 0·1N-NaOH. The mixture was extracted three times with ether; evaporation of the washed and dried ($Na_2SO_4$) ether left 'non-ketones' (17·00 g., after drying). The aqueous (ether-extracted) solution was left for some days after the addition of 10N-HCl (80 ml.). The (solid) precipitate was collected by filtration and the filtrate extracted twice with

a residue, which with 50% (v/v) light petroleum–ether gave a second crop (0·14 g., m.p. 110–126°) of crystals; evaporation of the final liquors gave a residual gum. The first crop (0·48 g.) of crystals was recrystallized from aqueous ethanol to give white glistening leaflets, m.p. 137·5–138·5°, of *ethyl 3α-hydroxy-6-oxoallocholanate.* (Found: C, 74·0; H, 10·1. $C_{26}H_{42}O_4$ requires C, 74·6; H, 10·0%.) Anchel & Schoenheimer (1938) give m.p. 136° but no analysis for this compound. This ester gave a colour on paper with the phosphomolybdic acid spraying reagent, but no spot after chromatography and spraying as described, with Bush's (1952) system *A*.

*Simplified separation.* The 'ethyl esters' (*n* g., where *n* was between 7 and 11) were dissolved in benzene (2–3*n* ml.) and poured on to a column (diam. 2·9 cm.) of $Al_2O_3$ (10*n* g.) made up in benzene. When the 'esters' had entered the column, they were eluted in the following order (fraction, approx. wt., solvent): fraction *A*, (0·35*n* g.), benzene (1 l.); fraction *B* (0·30*n* g.), ether (4 l.); fraction *C* (0·12*n* g.), acetone (500 ml.); fraction *D* (0·23*n* g.), ethanol

(500 ml.). Frequently a fraction (about 0·05*n* g.) remained on the column, presumably held as a salt, after hydrolysis by the $Al_2O_3$, and could be eluted with 1% (v/v) acetic acid–methanol. Fraction *A* was found to contain cholesterol and oily esters, fraction *B* was rich in ethyl chenodeoxycholate and contained ethyl hyodeoxycholate, fraction *C* was chiefly a mixture of ethyl hyodeoxycholate and ethyl hyocholate, and fraction *D* was very rich in ethyl hyocholate. Collected fraction *D* (3·8 g.) was boiled under reflux with ethanol (30 ml.) and 40% (w/v) aqueous KOH (4 ml.) for 1 hr. The cooled solution was diluted with water and acidified with 5N-HCl. After being kept at 5°, the solid precipitate was collected, washed, dried by evaporation *in vacuo* with ethanol and crystallized from ethyl acetate. Yield, 1·7 g. of crude hyocholic acid (containing hyodeoxycholic acid) of m.p. about 190°. For further purification, this material was esterified with ethanol by process *A* (above). The ethyl esters (1·7 g.) were dissolved in a mixture of acetic acid (10 ml.) and acetic anhydride (2 ml.). To the solution, cooled in a beaker of water, was added slowly 8·5N-$HClO_4$ (0·1 ml.). The warm mixture was gently agitated for a few moments and then left for 10 min. It was then diluted with water and extracted twice with ether. The ether extract was washed with water, aqueous $NH_3$, water, dried ($Na_2SO_4$) and evaporated. The residue with light petroleum gave at once thin prismatic crystals of crude triacetyl ethyl hyocholate, which were collected and washed with cold light petroleum; yield, 1·5 g. The mother liquors gave on evaporation a gum which, with light petroleum, slowly crystallized as needles of impure diacetyl ethyl hyodeoxycholate (Haslewood & Wootton, 1950), m.p. about 90°. The weight of crude triacetyl ethyl hyocholate obtained by this process could be taken as a good guide to the percentage of hyocholic acid present in a mixture (mainly) of this substance and hyodeoxycholic acid, since the triacetyl ethyl ester crystallized very easily and was sparingly soluble in light petroleum. This method was used to determine the percentage of hyocholic acid in specimens of crude 'hyodeoxycholic' acid, and in crystalline material directly obtained after hydrolysis of bile salts by methods (*a*) and (*b*) described above. The average yield of purified hyocholic acid was approximately 5% of the 'bile acids'.

*Hyocholic acid* (II). Purified triacetyl ethyl hyocholate (0·4 g.) was boiled under reflux with ethanol (15 ml.) and 40% (w/v) aqueous KOH (1 ml.) for 35 min. The cooled solution was acidified with 5N-HCl, and the solid precipitate collected and washed. The liquors on standing gave white needles, m.p. 183–185°, of hyocholic acid, and the main precipitate, after drying, was crystallized from ethyl acetate to yield colourless short white needles, m.p. 188–189°, of *hyocholic acid* (II), $[\alpha]_D^{21} + 5{\cdot}5 \pm 0{\cdot}5°$ in ethanol (*c*, 1·7), $[M]_D \simeq +22°$. (Found: C, 70·3; H, 9·7%; equiv. wt. (titration) 410. $C_{24}H_{40}O_5$ requires C, 70·6; H, 9·8%; equiv.wt., 408.)

*Properties of hyocholic acid.* Hyocholic acid resembled hyodeoxycholic acid in appearance and crystallized in a similar way as white needles from acidified dilute aqueous solutions of its salts. From ethyl acetate, the pure acid formed crystals very similar to, but less glistening than, those of pure hyodeoxycholic acid. The m.p. of an approximately 50% (w/w) mixture of hyocholic acid (m.p. 188–189°) and pure hyodeoxycholic acid (m.p. 197–198°) was 184–196°; such mixtures, but with about 20% (w/w) of hyocholic acid, crystallized as apparently homogeneous needles and melted sharply at about 190°. Hyocholic acid gave a negative Hammarsten HCl test and an orange-red colour in the Liebermann–Burchard test. It was sparingly soluble in ether and benzene, dissolved fairly readily in acetone and warm ethyl acetate and easily in ethanol or methanol. A cold solution of hyocholic acid (20 mg.) in acetic acid (0·5 ml.) was treated with 20% $CrO_3$ (4 drops), added gradually with shaking. A similar solution of hyodeoxycholic acid (20 mg.) was treated in exactly the same way. Both $CrO_3$-treated solutions were diluted and extracted with ether. The (water) washed and dried ($Na_2SO_4$) ether extracts were evaporated, and each residue was dissolved in N-NaOH (0·5 ml.) with water (1·5 ml.). Each solution was yellow-orange in colour, that from hyocholic acid being about twice as dark as the other. The product from hyocholic acid, precipitated by acidification, could not be induced to crystallize and did not give a crystalline ethyl ester (process *A*); after Kishner–Wolff reduction of it, no crystalline material could be isolated.

Hyocholic acid (24 mg.) was dissolved in dry acetone (1 ml.) by warming, and the cooled solution left at about 23° for 16 hr. after addition of a saturated solution of HCl gas in dry ether (1 drop). Aqueous 10N-HCl (1 drop) was added and the solution left for 24 hr. No precipitate formed and evaporation of solvents at about 25° *in vacuo* left a residue which did not crystallize even when 'seeded' with hyocholic acid. Mild acetylation (acetic anhydride–pyridine at room temp.) gave no crystalline material. As starting material could not be recovered after this mild treatment, acetonide formation might have occurred, at least partially.

### *Derivatives of hyocholic acid*

*Ethyl hyocholate.* This was prepared by process *A* and, crystallized from light petroleum–ether, formed short white needles, m.p. 76–78°. (Found, on a sample dried to const. wt. at 30°: C, 71·7; H, 10·1. $C_{26}H_{44}O_5$ requires C, 71·6; H, 10·1%.) Methyl hyocholate, similarly prepared, did not crystallize.

*Triacetyl ethyl hyocholate.* This was prepared by the $HClO_4$ method as described above (or, in inferior yield, by heating ethyl hyocholate for 2 hr. at about 95° in a mixture of equal vol. of acetic anhydride and pyridine), and when recrystallized from light petroleum–benzene formed large colourless crystals, very similar in appearance to those of cholesterol and having m.p. 185–187°; $[\alpha]_D^{22} + 27{\cdot}8 \pm 1°$ in ethanol (*c*, 1·2). (Found: C, 68·7; H, 9·0. $C_{32}H_{50}O_8$ requires C, 68·3; H, 8·9%.) Diacetyl ethyl hyodeoxycholate (Haslewood & Wootton, 1950) had $[\alpha]_D^{22} + 22{\cdot}3 \pm 1°$ in ethanol (*c*, 2·2). Triacetyl ethyl hyocholate was recovered unchanged after treatment in the cold with $CrO_3$ in acetic acid.

*Triacetyl methyl hyocholate.* This compound, similarly prepared and purified, resembled the corresponding ethyl ester in appearance. It had m.p. 184–185°; $[\alpha]_D^{22} + 28{\cdot}5 \pm 1°$ in ethanol (*c*, 1·1). (Found: C, 68·0; H, 8·8. $C_{31}H_{48}O_8$ requires C, 67·9; H, 9·0%.)

*Diacetyl ethyl hyocholate* (*probably ethyl 7α-hydroxy-3α:6α-diacetoxycholanate*, III). A solution of ethyl hyocholate (112 mg.) in dry pyridine (1 ml.) with acetic anhydride (1 ml.) was kept at about 23° for 16 hr. It was then diluted and extracted with ether. The ether was

washed with water, dried ($Na_2SO_4$) and evaporated. The residue was kept at about 5° with light petroleum or methanol, when it gradually formed large colourless cubical 'sugary' crystals (about 80 mg.); these were recrystallized from light petroleum–benzene and then gave (probably) *ethyl* 7α-*hydroxy*-3α:6α-*diacetoxycholanate* (III) of m.p. 121–123°, clearing at about 150°, depressed by ethyl 3α:6α-diacetoxy-7-oxocholanate (below). (Found: C, 68·85; H, 9·3. $C_{30}H_{48}O_7$ requires C, 69·2; H, 9·2%.) This compound was recovered substantially unchanged after: (i) keeping with freshly distilled toluene-*p*-sulphonyl chloride in pyridine or 2:4:6-collidine at about 20–25° for 4–5 days; or (ii) heating at 90–95° for 4 hr. with toluene-*p*-sulphonyl chloride in pyridine. Treatment of (III) with $POCl_3$ in pyridine gave unchanged material and gums; shaking with freshly sublimed $PCl_5$ in dry $CHCl_3$ with $CaCO_3$ gave a yellow product which could not be crystallized before or after treatment with $H_2$ and Adams Pt catalyst.

*Ethyl* 3α:6α-*diacetoxy*-7-*oxocholanate*. A solution of the above diacetate (III, 120 mg.) in acetic acid (1 ml.) was treated with 20% $CrO_3$ (0·1 ml.), added slowly with cooling and mixing. After 10 min. the product was diluted and extracted with ether. The ether was washed, dried ($Na_2SO_4$) and evaporated. The residue (115 mg.), which was recovered quantitatively in the 'non-ketonic' fraction after treatment with Girard's reagent 'T' in the usual way, was crystallized by the addition of light petroleum and recrystallized from light petroleum–benzene. *Ethyl* 3α:6α-*diacetoxy*-7-*oxocholanate* thus gave groups of small white needles (81 mg.), m.p. 121–123°. (Found: C, 69·2; H, 8·9. $C_{30}H_{46}O_7$ requires C, 69·5; H, 8·9%.) Three attempts by methods found successful in other cases were made to reduce this substance by the Kishner–Wolff process. All failed to yield crystalline material at any stage, and paper chromatography of the (ethylated) acids recovered from these experiments failed to give convincing evidence for the formation of hyodeoxycholic acid in them. A spot running rather faster than ethyl hyodeoxycholate was noticed, and an unsuccessful attempt to isolate an identifiable substance responsible for this was made by separation on paper, as previously applied (Haslewood, 1954*b*).

Clemmensen reduction: the ketone (0·1 g.) was added to a flask containing freshly amalgamated zinc wool (1 g. of Zn), water (0·75 ml.) and 10N-HCl (1·0 ml.). The flask was gently heated, under reflux, for 5·5 hr., during which time 10N-HCl (total 2·5 ml.) was added down the condenser at the rate of about 0·5 ml. every 30 min. The diluted cooled solution was extracted with ether and the ether washed with water, aqueous $NH_3$, water, dried ($Na_2SO_4$) and evaporated: the residue (59 mg.) was 'neutrals'. Acidification of the aqueous $NH_3$ washings gave 'acids' (19 mg.). Attempts were made to crystallize the (hydrolysed) 'neutrals' and also the 'acids' from ethyl acetate, but without success. Each fraction was then re-ethylated (process *A*) and examined by paper chromatography. The ethylated 'acids' gave no spots, but the re-ethylated 'neutrals' gave faint spots corresponding to ethyl hyocholate and ethyl hyodeoxycholate. The re-ethylated 'neutrals' (53 mg.) were dissolved in benzene, and the solution was poured on to $Al_2O_3$ (0·5 g., Hopkin & Williams Ltd., neutralized as described by Shoppee, 1949) in a column. Benzene (30 ml.) eluted a fraction (39 mg.), which crystallized from light petroleum and then from aqueous ethanol as short needles, m.p. 150–157°, tetranitromethane in $CHCl_3$, no colour. (Found: C, 75·0; H, 10·7. $C_{26}H_{44}O_4$ requires C, 74·3; H, 10·5%.) The nature of this material has not been further elucidated. No further crystalline substance could be isolated in this experiment. A second Clemmensen reduction, with acetic acid as solvent, gave, after similar processing, no crystalline material.

Hydrogenation: the ketone (40 mg.) in acetic acid (5 ml.) containing $H_2SO_4$ (0·2 ml.) was shaken with $PtO_2$ (50 mg.) in an atmosphere of $H_2$ at slightly increased pressure for 4 hr. Extraction of the product from the filtered and (aqueous) diluted mixture with ether gave, on evaporation of the washed ether, a residue, which was acetylated by the $HClO_4$ method. The only crystalline substance recovered after this process was an impure sample of triacetyl ethyl hyocholate.

3α:6α-*Dihydroxy*-7-*oxocholanic acid* (*acid A*, IV). The above ketone (88 mg.) was hydrolysed by boiling under reflux with a mixture of ethanol (2 ml.) and 40% (w/v) aqueous KOH (0·2 ml.). The cooled solution was diluted with water, acidified with HCl and treated with NaCl (excess). The solid precipitate was collected, washed with water and evaporated to dryness with ethanol *in vacuo*. The residue crystallized on adding ether, and the crystals were collected and washed with cold ether. Recrystallization from aqueous ethanol or light petroleum–ethanol gave 3α:6α-*dihydroxy*-7-*oxocholanic acid* (acid A, IV) as white needles which decomposed at about 211–215°, according to the rate of heating; $[\alpha]_D^{23}+12{\cdot}3\pm1°$ in ethanol (*c*, 0·8), $[M]_D \simeq +50°$. (Found: C, 70·6; H, 9·4. $C_{24}H_{38}O_5$ requires C, 70·9; H, 9·3%.)

(*Probably*) 3α:6α:7α:24-*tetrahydroxycholane, and its acetates*. Triacetyl ethyl hyocholate (0·2 g.) with dry ether (20 ml.) and powdered $LiAlH_4$ (0·2 g.) were gently heated under reflux for 2 hr. The mixture was poured onto ice, in aqueous HCl. The organic product was immediately extracted with ether and the ether washed with water, dried ($Na_2SO_4$) and evaporated. The (neutral) residue (131 mg.) formed gels with organic solvents, but crystals of m.p. about 140° were formed with light petroleum–ether; these were not further purified.

Tetraacetate: a solution of the $LiAlH_4$ product (64 mg.) in acetic acid (1 ml.) with acetic anhydride (0·2 ml.) was treated with 8·5N-$HClO_4$ (1 drop). After 10 min., the mixture was diluted with water and the separated solid collected, washed and dried with ethanol *in vacuo*. With light petroleum small white prisms appeared; these were recrystallized from dilute ethanol to give (probably) 3α:6α:7α:24-*tetraacetoxycholane*, m.p. 173–175°. (Found: C, 68·3; H, 9·1. $C_{32}H_{50}O_8$ requires C, 68·3; H, 8·9%.)

Triacetate: the $LiAlH_4$ product (68 mg.) was dissolved in dry pyridine (0·5 ml.) with acetic anhydride (0·5 ml.) and the solution kept at about 23° for 16 hr. It was then diluted with approx. N-HCl (excess) and the precipitate collected, washed and dried by evaporation *in vacuo* with ethanol. The residue crystallized with light petroleum as needles (70 mg.) of m.p. 115–118°; these were recrystallized from aqueous ethanol to give long white needles of (probably) 7α-*hydroxy*-3α:6α:24-*triacetoxycholane*, m.p. 114–116°; $[\alpha]_D^{20}+22{\cdot}5\pm1°$ in ethanol (*c*, 0·9). (Found: C, 68·7; H, 9·2. $C_{30}H_{48}O_7$ requires C, 69·2; H, 9·2%.) An attempt was made to dehydrate this substance (62 mg.) with freshly distilled $POCl_3$ (0·3 ml.) in dry pyridine (1 ml.) for 22 hr. at about 23°. No crystalline material could be isolated from the product, after careful chromatography on $Al_2O_3$.

3α:6α:24-*Triacetoxy*-7-*oxocholane*. The triacetate (30 mg.) in acetic acid (0·5 ml.) was treated at about 20° with 20% $CrO_3$ (2 drops). After 10 min., with occasional shaking, the solution was diluted with water and the solid precipitate collected, washed and dried by evaporation with ethanol *in vacuo*. The residue was crystallized from light petroleum and then from aqueous ethanol to give long white glistening needles of 3α:6α:24-*triacetoxy*-7-*oxocholane*, m.p. 130–131°; $[\alpha]_D^{19} + 21{\cdot}3 \pm 1°$ in ethanol (*c*, 0·75). (Found: C, 69·7; H, 9·1. $C_{30}H_{46}O_7$ requires C, 69·5; H, 8·9%.) This substance (194 mg.) was added to a suspension of powdered $LiAlH_4$ (0·2 g.) in dry ether (20 ml.) and the mixture was gently boiled under reflux for 2 hr. The $LiAlH_4$ product was worked up as described above and was then acetylated by the $HClO_4$ method and separated by chromatography. The above tetraacetate (110 mg.) and an impure sample (about 5 mg.) of a different compound could be isolated. 3α:6α:24-Triacetoxy-7-oxocholane (10 mg.) was gently heated under reflux at intervals for 5 days with 50% (v/v) ethanol–water (5 ml.), semicarbazide hydrochloride (0·1 g.) and hydrated sodium acetate (0·1 g.). A few crystals, decomp. about 260°, were formed, but these were also obtained with semicarbazide and sodium acetate alone. Dilution of the reaction mixture (after 5 days) gave crystals (5–8 mg.) of m.p. about 120°, which did not contain nitrogen. An attempt at Kishner–Wolff reduction of the above ketone (39 mg.) gave a product (32 mg.) from which, after acetylation and chromatography, no identifiable material could be isolated.

*Thilobilianic acid* (V). A solution of ethyl hyocholate (0·2 g.) in acetic acid (2 ml.) was kept at about 20° by cooling in water and treated, with shaking, with 20% $CrO_3$ (1 ml.) added gradually from a burette during 5 min. After 3·5 hr. at 20°, with occasional shaking, the solution was diluted and shaken twice with ether. Insoluble material which separated was dissolved in aqueous $NH_3$, which was used to extract the ether. The ether was then washed with water and all the aqueous $NH_3$ and water washings were combined and treated with 5*N*-HCl (excess) and NaCl (excess). The mixture was warmed gently and surplus ether was expelled by blowing $N_2$ gas on to the surface. The gummy solid which separated was collected, washed and stirred with dilute aqueous $NaHCO_3$. The filtered solution was acidified with HCl and the precipitate collected, washed with water and dried by evaporation *in vacuo* with ethanol, giving gummy 'acids' (120 mg.). Evaporation of the above washed ether gave 'neutrals' (32 mg.); this material could not be induced to crystallize, and treatment of it by the Kishner–Wolff method gave no crystalline product.

The 'acids' (120 mg.) were transferred in ethanol to a small metal bomb and solvent was removed with $N_2$. The residue was dissolved in ethanol (4 ml.), to which was added sodium (0·25 g.) and '100%' hydrazine hydrate (0·4 ml.). The bomb was sealed and heated at 190–200° for 4 hr., and then cooled. The contents were diluted with water. Acidification of the filtered solution gave a white solid, which was collected, washed with water and dried by evaporation *in vacuo* with ethanol. The residue was crystallized from ethyl acetate to give thilobilianic acid (V) as small white silky needles, m.p. 255–257° (decomp.). (Found: C, 68·0; H, 8·8%; equiv.wt. (titration) 141. Calc. for $C_{24}H_{38}O_6$: C, 68·6; H, 9·05%; equiv.wt. 144.) Yield, about 25 mg. Wieland & Dane (1932) give m.p. 260–262° (decomp.) for this substance.

*Preparation of reference compounds*

3α:6α-*Dihydroxy*-7-*oxocholanic acid* (*acid A*, IV). Ethyl 3α-acetoxy-7-oxocholanate (Haslewood, 1954*b*; from 0·1 g. of 3α-hydroxy-7-oxocholanic acid) was dissolved at about 20° in 1 ml. of a freshly made solution of bromine (0·1 ml.) in acetic acid (7·8 ml.). To this was added 1 drop of a solution of HBr in acetic acid, and the stoppered mixture was left at about 20° for 16 hr.; bromine disappeared. Solvent was evaporated at about 30° *in vacuo*, and to the residue were added KOH (approx. 0·3 g.) and methanol (4 ml.). The solution was gently boiled under reflux for 1 hr., and was then cooled, diluted with water and acidified with HCl. Addition of NaCl (excess) gave a solid which was collected, washed with water and evaporated with ethanol to dryness *in vacuo*. A solution of the residue in ethyl acetate was filtered and evaporated to about 1 ml. Crystals separated in two forms: needles (m.p. 201–206° decomp.) and large prisms (m.p. about 225°). The needles, which were in much larger amount, were collected separately and recrystallized from light petroleum–ethyl acetate. Needles, decomp. range about 211–214°, were obtained, and these did not depress the m.p. of a sample of (IV) from hyocholic acid (above). A sample (1 mg.) of (IV) from each source was methylated with diazomethane in ether and solvent removed. The residues were examined by infrared spectroscopy by Dr I. D. P. Wootton, who reported that the spectra were identical. Evaporation of solvent ($CS_2$) used for the infrared analysis left crystalline samples of the methyl esters; the crystals were washed with light petroleum. The crude products melted as follows: from hyocholic acid, at 110–115°; from 3α-hydroxy-7-oxocholanic acid, at 114–117°; mixed m.p. 110–115°. The substance (IV) was also obtained (by bromination and alkaline hydrolysis of the product) from 3α-acetoxy-7-oxocholanic acid, prepared *in situ* by acetylation of 3α-hydroxy-7-oxocholanic acid.

3α:6α:24-*Triacetoxycholane* (VI). To a solution of ethyl hyodeoxycholate (0·1 g.) in dry ether (10 ml.) was added powdered $LiAlH_4$ (0·1 g.), and the mixture was gently boiled under reflux for 1 hr. It was then cooled and poured into HCl–ice. The organic material was extracted with ether and the ether washed with water, aqueous $NH_3$, water, dried ($Na_2SO_4$) and evaporated. The residue, from light petroleum–ether, gave large crystals of m.p. about 170°, which appeared to be hydrated. (Found: C, 75·4; H, 11·1. $C_{24}H_{42}O_3$ requires C, 76·2; H, 11·1%.) This material (30 mg.) was dissolved in pyridine (1 ml.) with acetic anhydride (1 ml.) and the mixture kept at about 25° for 24 hr.; it was then diluted with aqueous HCl (excess). The precipitate was collected, washed with water, evaporated to dryness with ethanol *in vacuo* and crystallized twice with light petroleum–benzene, giving white needles of 3α:6α:24-*triacetoxycholane* (VI), m.p. 129–131°. (Found: C, 71·1; H, 9·5. $C_{30}H_{48}O_6$ requires C, 71·4; H, 9·5%.)

*Ethyl* 3β:6α-*dihydroxycholanate*. 3β:6α-Dihydroxycholanic acid, prepared from pig gallstone (Haslewood & Wootton, 1950), was ethylated by process *A*: the product crystallized readily from light petroleum–benzene in short white needles of *ethyl* 3β:6α-*dihydroxycholanate*, m.p. 131–134°. (Found: C, 74·6; H, 10·2. $C_{26}H_{44}O_4$ requires C, 74·3; H, 10·5%.) This substance did not give a spot on paper chromatography as described with Bush's (1952) system *A*.

## DISCUSSION

*Chemical.* The present proof of the structure of hyocholic acid rests largely on the correctness of formula (IV) for the acid A, obtained by alkaline hydrolysis of brominated 3α-acetoxy-7-oxocholanic acid or its ethyl ester.

Wieland & Dane (1932) and Dane & Wulle (1941) brominated 7-oxocholanic acid. They obtained a product in which the bromine atom was shown to be at C-6 and probably β-orientated. Lardon (1947) brominated methyl 3α-succinyloxy-7-oxoaetianate and heated the crude product with methanolic sodium hydroxide. The acid so obtained was converted into a substance described as methyl 3α:6α-diacetoxy-7-oxoaetianate; Kishner–Wolff reduction of this, followed by further treatment, gave methyl 3:6-dioxo*allo*aetianate in poor yield. More recently, Takeda, Igarashi & Komeno (1954*a*) actually brominated methyl 3α-acetoxy-7-oxocholanate. The principal product was resistant to debromination and hence the bromine atom (at C-6) was probably β-orientated (*cis* elimination as HBr with H at C-5 very difficult). Hot saponification (Takeda, Komeno & Igarashi, 1954*b*) of the bromo derivative gave an acid having m.p. 227° which may well be identical with acid A (IV), for the m.p. (decomposition point) of A is variable, according to the rate of heating. The acid of m.p. 227° is thus probably (IV), Walden inversion at C-6 having occurred, as expected, during hydrolysis. Takeda *et al.* (1954*b*) suggested 3α:7-dihydroxy-6-oxo*allo*cholanic acid for the structure of their acid, but provided no evidence for this formula. By cold saponification of their (presumably) methyl 3α-acetoxy-6β-bromo-7-oxocholanate, Takeda *et al.* derived an acid of m.p. 185°, to which they assigned formula (IV) and which they converted into hyodeoxycholic acid; an explanation of these findings is not at present obvious.

This prior work tends on the whole to support the structure (IV) for acid A.

Thilobilianic acid (V) was made by Wieland & Dane (1932) by oxidation of 7-hydroxy- and 7-oxo-cholanic acids, and may be assumed to have the hydrogen atom at C-5 in the β position; hence its formation from hyocholic acid proves that this substance too has the 5β (cholane) structure.

Examination of molecular models suggests that the reactions of a 7α-hydroxyl group in a cholane compound would be hindered, and that such hindrance would be greatly increased by a hydroxyl or acetoxy group at C-6α. 7α-Hydroxylation also might be expected on biogenetic grounds, in the present case. Such considerations do not, however, amount to proof of configuration.

Hyocholic acid is thus satisfactorily proved to be a 3α:6α:7-trihydroxy- and is probably 3α:6α:7α-trihydroxy-cholanic acid (II); the 'hindered' group is the one at C-7.

This appears to be the first authentic example of a 6:7-dihydroxycholane, although 6-hydroxy-7-oxo and 7-hydroxy-6-oxo compounds of this type are well known. The remarkable feature of the 6:7-dihydroxycholane now discovered is the enhancement of the (somewhat) hindered nature of the C-7-hydroxyl group by the neighbouring C-6α-hydroxyl (or -acetoxy) group. This C-7 group was more difficult to acetylate than a C-12α-hydroxyl group and could not (when –OH at C-6 was acetylated) be induced to give a toluene-*p*-sulphonyl derivative. The corresponding C-7 ketone (acetoxy at C-6; >C=O at C-7) was entirely inert to Girard's reagent and semicarbazide.

It is of course well known that a C-7α-hydroxyl group in cholane derivatives is a little hindered in esterification reactions, and that the corresponding ketone, although it forms a semicarbazone (Haslewood, 1943), does not quantitatively react with Girard's reagent (Hoehn, Schmidt & Hughes, 1944; Haslewood, 1954*b*); nevertheless, the increased degree of 'hindering' at C-7 observed in hyocholic acid and its derivatives was quite unexpected and accounted for much of the difficulty of the present investigation.

Although its conjugate was not isolated from paper chromatograms, the general behaviour of hyocholic acid and its esters leaves little doubt that its glycine conjugate was in fact the substance observed by Haslewood & Sjövall (1954) and mentioned above.

Trickey's (1950) acid '$C_{27}H_{44}O_6$' was isolated only as the azoylamide of indefinite melting point; his formula, based only on elementary analysis, might be explained if his material was hydrated hyocholic acid azoylamide. The percentage (6 %) of pig bile acids in this fraction would agree with the findings for hyocholic acid now recorded. The point is an important one, as no mammal has so far been found to secrete a $C_{27}$ bile acid: it would therefore be of great interest if the occurrence of such a compound could be confirmed.

*Biological.* As a working hypothesis, hyocholic acid in the pig can be considered as corresponding to cholic acid in other mammals, i.e. where these have 3α:7α:12α-trihydroxy-cholanic (cholic) acid, the pig has 3α:6α:7α-trihydroxycholanic (hyocholic) acid.

The dog can make cholic acid from cholesterol, and the rat has been shown to make its chief bile acids (cholic and chenodeoxycholic) from the same source (for references, see Haslewood, 1955). The rabbit converts cholesterol into deoxycholic (3α:12α-dihydroxycholanic) and cholic acids (Ekdahl & Sjövall, 1955). Similarly, the pig may make from cholesterol the bile acids mentioned (above) as occurring in its bile.

The pig is not known to form deoxycholic or cholic acids, or any bile acid hydroxylated at C-12. No other mammal has so far been shown to make (normally) any bile salt hydroxylated at C-6; the only known examples of creatures having this ability are certain teleostean fish and the domestic hen (Ohta, 1939; Isaka, 1940; Isaka & Azato, 1940; Mabuti, 1941; Yamasaki, 1951).

Thus pig bile salts are unique in a special way. This uniqueness must be due to (*a*) mutation occurring in ancestors having already the cholesterol → cholic acid sequence (or part of it), or (*b*) descent from a very primitive form originally making C-6-hydroxylated bile salts. A decision between possibilities (*a*) and (*b*) obviously cannot be made except by consideration of all the available evidence, but when the evolutionary position of the pig is ascertained its relationship to that of other vertebrates will have to account for the remarkable chemical nature of pig's bile salts. In considering this question, it should be noted that the general trend of the present work suggests that the chemical nature of bile salts is of a very conservative character.

## SUMMARY

1. Hyocholic acid, $C_{24}H_{40}O_5$, from pig bile has been shown to be 3α:6α:7-trihydroxycholanic acid (II); the hydroxyl group at C-7 is probably α-orientated.

2. This substance appears to be the first authentic example of a 6:7-dihydroxycholane: the presence of the hydroxyl group at C-6 in this cholane derivative causes marked hindrance in the reactions of the C-7 hydroxyl group and of the ketone derived from it.

3. Pig bile contains at least one unidentified substance, giving a spot on paper chromatograms of the ethylated 'bile acids'.

4. Attention is drawn to the remarkable chemical nature of pig bile salts, and it is suggested that this unique nature must be taken into consideration in studies of pig evolution.

The author wishes to thank Mr A. Baker for collections of fresh pig bile, and Dr I. D. P. Wootton for the infrared spectral examination.

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# Experiments on the Lysine and Aspartic Acid Residues in Bacitracin A

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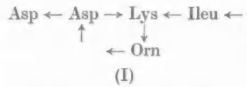
(*Received* 19 *September* 1955)

In several structural features the bacitracin family appears to be unique among polypeptides that have so far been subjected to detailed chemical study. The *N*-terminal and sulphur-containing residues in bacitracin A have been discussed in an earlier paper (Lockhart, Abraham & Newton, 1955). In addition to this portion of the molecule, the lysine residue is a centre of interest.

The earliest experiments on the reaction of bacitracin A with 1-fluoro-2:4-dinitrobenzene (FDNB) revealed that neither of the two amino groups of the lysine residue in the molecule was free (Craig, Hausmann & Weisiger, 1952; Newton & Abraham, 1953). Studies of the amino acid sequence in bacitracin A showed that the probable arrangement of residues in the vicinity of the lysine was

that shown below (I), in which → denotes a C–N bond (Craig, Haussmann & Weisiger, 1954; Lockhart & Abraham, 1954*a*).




(I)

Some of the simple peptides containing lysine that were obtained from bacitracin A were remarkably resistant to hydrolysis, surviving prolonged treatment with 11N hydrochloric acid at 80° (Lockhart & Abraham, 1954*b*). The results of experiments that were made possible by this stability are described in the present paper.

## METHODS

### *Bacitracin used for hydrolysis*

*Bacitracin A.* The sample of bacitracin A was that described by Lockhart & Abraham (1954*a*).

*Commercial bacitracin.* Commercial bacitracin (lot 25, 61 i.u./mg. and lot B-55–13, 68 i.u./mg.) was supplied by the Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A.

*Deaminated bacitracin A.* This was prepared by treating bacitracin A with nitrous acid as described by Lockhart *et al.* (1955).

*Desulphurized bacitracin.* This was prepared by treating commercial bacitracin with Raney nickel as described by Lockhart *et al.* (1955).

### *Partial hydrolysis and separation of peptides*

*Hydrolysis.* Samples of bacitracin were hydrolysed in 11N-HCl (20–40 mg./ml.) in sealed tubes at 80° for times varying from 21 to 91 hr. The hydrolysates were then evaporated to dryness *in vacuo*.

*Ionophoresis in a four-compartment cell.* In some experiments a preliminary separation of the acidic, neutral and basic material in hydrolysates was made in a four-compartment cell similar to that described by Synge (1951).

*Ionophoresis on paper.* Peptides were separated by ionophoresis on sheets of Whatman no. 1 paper (36 cm. long) and eluted from the paper in the manner described by Lockhart & Abraham (1954*a*). Collidine–acetate buffer, pH 7, was used (Newton & Abraham, 1954) and the potential gradient was 14v/cm. Ionophoresis was generally carried out for 8 hr., but in some analytical experiments the time was shortened to 2·5 hr. The spots were located by spraying with ninhydrin.

*Paper chromatograms.* Peptides were also separated by chromatography on Whatman no. 1 paper, with butanol–acetic acid (Woiwod, 1949) as solvent system. The peptides were eluted from the paper in the manner described by Lockhart & Abraham (1954*a*).

### *Analysis of peptides*

Peptides which appeared to be homogeneous were hydrolysed in 6N-HCl at 110° for 20 hr. Qualitative analysis of the resulting amino acids was made by chromatography and ionophoresis on paper. Isoleucine was distinguished from leucine on chromatograms run with the *tert.*-pentanol–water system of Work (1949). Lysine was distinguished from ornithine by ionophoresis under the conditions described by Lockhart & Abraham (1954*a*). The other amino acids encountered could be distinguished on chromatograms run with butanol–acetic acid (Woiwod, 1949).

The *N*-terminal amino acids in the peptides were determined by the method of Sanger & Thompson (1953), the experiments being carried out under the conditions used by Lockhart & Abraham (1954*a*). The ether-soluble dinitrophenyl (DNP)-amino acids were run on paper chromatograms, the system of Blackburn & Lowther (1951) being used.

*Separation of α-DNP-lysine and ε-DNP-lysine.* The water-soluble α-DNP-lysine and ε-DNP-lysine could easily be distinguished by ionophoresis on paper in 0·02M sodium borate buffer, pH 9·1. A potential gradient of 14v/cm. was applied to the paper for 16 hr. At the end of the experiment α-DNP-lysine had migrated 9·3 cm. towards the cathode and ε-DNP-lysine 4·9 cm. towards the cathode. At pH 9·1 both these substances would be expected to carry a net negative charge. Their migration towards the cathode is presumably due to the endosmotic flow in the system used. However, the p*K* of the free amino group of α-DNP-lysine should be higher than that of ε-DNP-lysine, because the amino group in the former compound is further removed from the influence of the carboxyl group. Hence the net negative charge of α-DNP-lysine should be less than that of ε-DNP-lysine and the former should be carried further towards the cathode. A similar phenomenon was demonstrated with leucine and 6-aminohexanoic acid in the same system. Leucine migrated 8 cm. and 6-aminohexanoic acid 10 cm. towards the cathode.

### *Optical configuration of aspartic acid residues*

*Estimation of aspartic and glutamic acids.* The amounts of free aspartic and glutamic acids in bacitracin A that had been hydrolysed with 11N-HCl at 80° for 43 hr. were determined on a column of Dowex 50 × 8 (100 cm. × 0·9 cm.) by the procedure of Moore & Stein (1951). About 4·5 mg. of hydrolysate was applied to the column. The experiment was terminated after the emergence of glutamic acid.

D-*Aspartic acid oxidase.* This was prepared as described by Still, Buell, Knox & Green (1949). The kidneys from three rabbits yielded 2·5 g. of an acetone-dried powder. The enzyme solution used in the following experiments was prepared by stirring the powder with 10 parts by weight of 0·1M sodium phosphate buffer, pH 7·2, and removing the solid by centrifuging.

*Uptake of oxygen by aspartic acid in the presence of* D-*aspartic acid oxidase.* This was measured with a Warburg respirometer at 37°. The oxidation was carried out in the presence of ethanol, since under these conditions the rate of $O_2$ uptake is about doubled (Keilin & Hartree, 1936; Still *et al.* 1949). The main compartment of each vessel contained 1 ml. of enzyme solution, 0·4 ml. of 0·1M sodium phosphate buffer, pH 7·2, and 0·4 ml. of 4% (v/v) ethanol. The centre well contained 0·2 ml. of 10N-NaOH. The sample of the amino acid (1–3 mg.) in 0·4 ml. of 0·1M sodium phosphate buffer, pH 7·2, was placed in the side arm. The vessels were filled with $O_2$.

Under the conditions used about 50 $\mu$l. of $O_2$ was absorbed in 70 min. in the presence of 1 mg. of DL-aspartic acid, 100 $\mu$l. in the presence of 2·0 mg. and 140 $\mu$l. in the presence of 3·0 mg. The $O_2$ uptake after 70 min. was used to estimate the amount of D-aspartic acid in mixtures of aspartic acid (1·5–3·0 mg.) and glutamic acid (0–1·5 mg.) obtained from bacitracin. No absorption of oxygen occurred in the presence of L-aspartic acid (3 mg.) or DL-glutamic acid (3 mg.).

*Isolation of aspartic and glutamic acids.* Aspartic and glutamic acids were isolated from hydrolysates obtained by treating bacitracin (5 g.) with 11 N-HCl at 80° and 6 N-HCl at 110°. After removal of the HCl *in vacuo* a preliminary fractionation of the hydrolysate was carried out by ionophoresis in the four-compartment cell. The acidic fraction (200–700 mg.) was chromatographed on a column (1·5 cm. × 34 cm.) of Dowex 1 × 10 acetate in 0·5 N acetic acid (cf. Hirs, Moore & Stein, 1954). The effluent was collected in 2 ml. fractions with a flow rate of 8 ml./hr. Samples (10 $\mu$l.) were removed from every third fraction for the determination of ninhydrin colour densities (Moore & Stein, 1954). The fractions containing glutamic acid (usually 22–36) were combined and concentrated in a rotary evaporator (Craig, Gregory & Hausmann, 1950) to about 2 ml., when the product crystallized. Ethanol (6 ml.) was added, the mixture cooled in ice and the glutamic acid removed by filtration. The fractions containing aspartic acid (usually 90–130) were treated similarly. The products behaved like pure glutamic acid and aspartic acid respectively when chromatographed on paper in butanol–acetic acid.

## RESULTS

### *Peptides from bacitracin A*

The results obtained when samples of bacitracin A that had been hydrolysed with 11 N hydrochloric acid at 80° for periods varying from 21 to 91 hr. were examined by ionophoresis on paper at pH 7 are shown in Fig. 1. It is clear that large amounts of the free acidic and basic amino acids were liberated under the conditions of hydrolysis, with the notable exception of lysine. Even after hydrolysis for 43 hr. there appeared to be no significant quantity of free lysine present. Only three spots which could be assigned to peptides were then detected by ionophoresis on paper (Fig. 1, spots 1–3). A fourth peptide, which was neutral, was revealed by chromatography on paper in butanol–acetic acid, when it showed an $R_F$ value greater than that of leucine.

When the time of hydrolysis varied from 21 to 31 hr., two spots, other than 1–3 and those due to free amino acids, were revealed by ionophoresis on paper (Fig. 1). The positions of these spots (cf. Lockhart & Abraham, 1954*a*) suggested that the one between histidine and spot 3 was due to Orn.Ileu.Phe, and that the one between the neutral material and glutamic acid was Leu.Glu.

An analysis of peptides 1–4 is given in Table 1, the results being recorded in the manner used by Lockhart & Abraham (1954*a*). Peptides 1–3 were separated by ionophoresis on paper and peptide 4 was eluted from a paper chromatogram developed with butanol–acetic acid. The peptides appeared to be Asp (Ileu).Lys, Asp.Lys, [Ileu, Lys] and Ileu.Phe respectively. The finding of Hausmann, Weisiger & Craig (1955) that Ileu.Phe (peptide 4) gives a poor colour yield with ninhydrin was confirmed.

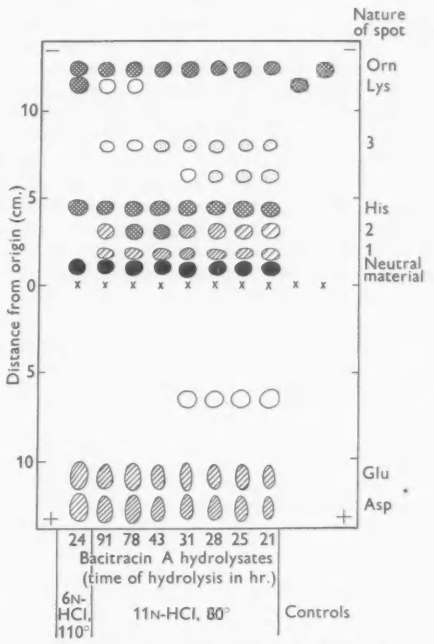



Fig. 1. Ionophoresis on paper at pH 7 for 2·5 hr. of products formed from bacitracin A in 11 N-HCl at 80°. The depth of shading represents the approximate intensity of the spots obtained when the paper was sprayed with ninhydrin.

### *Peptides from deaminated bacitracin A*

An experiment in which a sample of deaminated bacitracin A was treated with 11 N hydrochloric acid at 80° for 43 hr. yielded the four peptides that were obtained under similar conditions from bacitracin A. The phenylalanine obtained by hydrolysis of peptide 4, however, was accompanied by a small quantity of material which gave an unidentified grey-blue spot ($R_F$, 0·69 in butanol–acetic acid). The colour of the spot was similar to that given by phenylalanine with ninhydrin. A similar spot was obtained in earlier work on the amino acid sequence in bacitracin A (Lockhart & Abraham, 1954*a*).

After elution from a chromatogram and hydrolysis with 6N hydrochloric acid at 110° the substance responsible for this spot appeared to be unchanged, indicating that it was not a peptide.

*Peptides from commercial bacitracin*

*Commercial bacitracin.* Partial hydrolysis of bacitracin (lot 25) in 11N hydrochloric acid at 80° for 43 hr. again yielded peptides 1–4. In this experiment, a sufficient amount of peptide 3 (Ileu.Lys) was obtained to carry out an analysis for free amino groups. After hydrolysis of the DNP-peptide with 6N hydrochloric acid at 110° for 9 hr. in a sealed tube under nitrogen, ε-DNP-lysine was identified in the water-soluble material and the ether-soluble fraction yielded DNP-isoleucine. The ether-soluble fraction, unlike the corresponding fraction from peptides 1 and 2 (Table 1), showed no green fluorescent spot at the origin on paper chromatograms which were run under the conditions described by Blackburn & Lowther (1951) and viewed under ultraviolet light (365 mμ.).

Table 1. *Peptides from bacitracin A after hydrolysis with* 11N *hydrochloric acid at* 80° *for* 43 *hr.*

| Spot no. | $R_F$ value (butanol–acetic acid) | Migration (pH 7·0, 8 hr.) (cm.) | Amino acids present | Strength of amino acid after Hydrolysis | Strength of amino acid after DNP treatment | DNP-amino acid identified | Structure |
|---|---|---|---|---|---|---|---|
| 1* | 0·42 | −6·0 | Lys | + + + + | + + + | Ileu | Asp.(Ileu).Lys |
| | | | Asp | + + + + | + | Asp† | |
| | | | Ileu | + + + + | · | | |
| 2 | 0·15 | −7·75 | Asp | + + + + | + | Asp† | Asp.Lys |
| | | | Lys | + + + + | + +‡ | | |
| 3 | 0·39 | −14 | Ileu | + | · | · | [Ileu, Lys] |
| | | | Lys | + | · | | |
| 4 | 0·72 | −2·0 | Ileu | + + + | + | Ileu | Ileu.Phe |
| | | | Phe | + + + | + + + | | |

* This spot gave a brown colour with ninhydrin.

† The DNP-Asp was accompanied by material which remained at the origin on paper chromatograms (Blackburn & Lowther, 1951) and showed a bright-green fluorescence in ultraviolet light (365 mμ.).

‡ Identified as α-DNP-Lys.

*Desulphurized bacitracin.* The peptides Asp.(Ileu).Lys and Asp.Lys were also identified in partial hydrolysates of a sample of bacitracin (lot 25) that had been desulphurized by treatment with Raney nickel.

*Optical configuration of the two aspartic acid residues in bacitracin*

Bacitracin A contains one residue of glutamic acid and two residues of aspartic acid. Analysis on a column of Dowex 50 (Moore & Stein, 1951) of a hydrolysate obtained by treating bacitracin A with 11N hydrochloric acid at 80° for 43 hr. showed that at least 0·9 mole of glutamic acid, but only 1·34 moles of aspartic acid, were present in the free state. The work already described indicated that the remaining bound aspartic acid, which could be liberated by further hydrolysis with 6N hydrochloric acid at 110°, consisted almost entirely of the residue linked directly to lysine. A preliminary experiment was carried out in which D-aspartic acid oxidase was used to determine the proportion of D- and L-isomers in both the aspartic acid liberated by hydrolysis at 80° and in that liberated subsequently by hydrolysis at 110°. In a second experiment two samples of aspartic acid were isolated in crystalline form, and their specific rotations were measured.

*Use of* D-*aspartic acid oxidase.* Bacitracin (88 mg. of lot 25) was dissolved in 10 ml. of 11N hydrochloric acid and the solution kept at 80° for 43 hr. After removal of the hydrochloric acid *in vacuo* the hydrolysate was subjected to ionophoresis in a four-compartment cell. The acidic portion, consisting largely of free aspartic and glutamic acids, was named fraction A. The rest of the material, containing peptides of aspartic acid and lysine, was hydrolysed with 6N hydrochloric acid at 110° for 24 hr. The acidic material in this hydrolysate consisted mainly of aspartic acid. After separation from the neutral and basic material by ionophoresis in the four-compartment cell it was named fraction B.

A further sample of bacitracin (76 mg. of lot 25) was hydrolysed with 3 ml. of 6N hydrochloric acid at 110° for 24 hr. The acidic amino acids (known from earlier work to consist of 1 mole of glutamic acid and 2 moles of aspartic acid) were separated from the remainder by ionophoresis in the four-compartment cell and the resulting mixture was named fraction C.

The total amino acid content of each of the fractions A–C was estimated from photometric ninhydrin determinations (Moore & Stein, 1948). When considered, together with the proportions in

which the two acidic amino acids were known to be liberated from bacitracin A on hydrolysis under the conditions used, these determinations indicated that the three fractions had the following composition: fraction A, 1·5 mg. of aspartic acid plus 1·0 mg. of glutamic acid; fraction B, 1·5 mg. of aspartic acid; fraction C, 3 mg. of aspartic acid plus 1·6 mg. of glutamic acid. The amount of D-aspartic acid in each fraction was estimated by comparing its rate of oxidation, in the presence of D-aspartic acid oxidase, with the rates of oxidation of control solutions containing known amounts of D-aspartic acid (see Methods). The results indicated that the aspartic acid in fraction A was $66 \pm 2\%$ D, whereas that in fraction B was $58 \pm 2\%$ L and that in fraction C was racemic.

*Isolation of two samples of aspartic acid.* Bacitracin (5 g. of lot B 55–13) was hydrolysed in 125 ml. of 11N hydrochloric acid. Two fractions containing aspartic acid (corresponding to A and B of the preceding experiment) were isolated by a procedure similar to that already described. In the present case, however, fraction A consisted of acidic material liberated on hydrolysis for 20 hr. (instead of for 43 hr.) at 80°. After removal of fraction A, the remaining neutral material was hydrolysed for a further 24 hr. at 80°. The acidic material formed during this time was removed, by ionophoresis in the four-compartment cell, and discarded. The remainder of the sample was hydrolysed in refluxing 6N hydrochloric acid (190 ml.) for 24 hr., and the acidic material liberated during the hydrolysis represented fraction B. In a parallel experiment, 5 g. of bacitracin was first treated with 11N hydrochloric acid at 80° for 44 hr. and then with refluxing 6N hydrochloric acid for 24 hr. The acidic portion of the final hydrolysate represented fraction C.

The aspartic and glutamic acids in fractions A and C and the aspartic acid in fraction B (which contained almost no glutamic acid) were isolated after chromatographing the fractions on columns of Dowex 1 acetate (see Methods). The amounts of crystalline amino acids that were obtained, and the specific rotations of these amino acids in N hydrochloric acid, are shown in Table 2. The results indicate that the aspartic acid in fraction A is 93% D, and that in fraction B is 76% L. The aspartic acid in fraction C is almost completely racemic.

The fact that bacitracin A yields D-glutamic acid on acid hydrolysis (see Table 2) has been reported previously. Glutamic acid which was isolated from bacitracin A that had been hydrolysed with 6N hydrochloric acid at 110° for 24 hr. showed $[\alpha]_D - 25°$ in N-HCl (Craig *et al.* 1952).

Table 2. *Specific rotations of aspartic and glutamic acids liberated from bacitracin under various conditions of hydrolysis*

The yields represent the amounts of amino acids isolated from 5 g. of bacitracin. Optical rotations were measured in N-HCl (c, 3·0). For details of hydrolysis and isolation see text. Fischer (1899) gives: D-aspartic acid, $[\alpha]_D^{20} - 25·5°$ in 0·97N-HCl; D-glutamic acid, $[\alpha]_D^{20} - 30·05°$ in 0·37N-HCl.

| Fraction from hydrolysate | Aspartic acid | | Glutamic acid | |
|---|---|---|---|---|
| | Yield (mg.) | $[\alpha]_D^{20}$ | Yield (mg.) | $[\alpha]_D^{20}$ |
| A | 144 | $-22 \pm 0·5°$ | 159 | $-29 \pm 0·5°$ |
| B | 151 | $+13·5 \pm 0·5°$ | — | — |
| C | 512 | $-1 \pm 0·5°$ | 274 | $-23 \pm 0·5°$ |

## DISCUSSION

With the exception of Ileu.Phe, which was first found by Barry, Gregory & Craig (1948) in hydrolysates of bacitracin A, all four peptides that were detected after treating bacitracin A with 11N hydrochloric acid at 80° for 43 hr. contained lysine. The small number of these peptides, and the fact that they differed sharply in isoelectric point or in size, made their separation by ionophoresis on paper at pH 7 a relatively simple matter. The formation of the three peptides containing isoleucine from deaminated bacitracin A, as well as from bacitracin A itself, confirms the conclusion (Lockhart & Abraham, 1954*b*) that neither the isoleucine residue linked to lysine nor that linked to phenylalanine occupies an *N*-terminal position.

The three peptides containing lysine behaved as though they were Asp(Ileu).Lys, Ileu.Lys and Asp.Lys respectively. The most resistant to hydrolysis by acid was Asp.Lys. The stability of the peptides may be due to the presence in them of two positively charged amino groups and of amino acids with large side chains. Both factors might hinder the approach of the hydroxonium ion which catalyses the hydrolysis of the peptide bond.

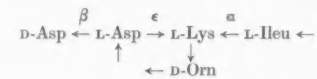
The separation of two dipeptides containing lysine as a *C*-terminal amino acid enabled the residues linked to each of the two amino groups of lysine to be determined by the use of FDNB. The experiments appeared to show clearly that an isoleucine residue was joined to the α-amino group of lysine and an aspartic acid residue to the ε-amino group.

The work of Craig *et al.* (1952) had shown that when all the aspartic acid was liberated from bacitracin A by hydrolysis with 6N hydrochloric acid at 110° the resulting product was racemic. The simplest explanation of this finding was that one residue had the D- and one the L-configuration. The picture obtained when samples of bacitracin A which had been treated with 11N hydrochloric acid at 80° for varying periods of time were subjected to ionophoresis on paper at pH 7 indicated

that one of the aspartic acid residues rapidly appeared as free aspartic acid. Since the second aspartic acid residue was found to be linked to lysine by an unusually stable bond it seemed possible that two aspartic acids could readily be separated from each other. In the first experiment that was made, in which the time of the primary hydrolysis was 43 hr., the amount of free aspartic acid formed was 66% of the total amount in bacitracin A. The easily liberated aspartic acid obtained under these conditions was therefore mixed with some of the aspartic acid linked directly to lysine, a finding in accordance with the fact that the peptide Ileu.Lys was present in the hydrolysate. The behaviour of the material towards D-aspartic acid oxidase indicated that about 66% of the aspartic acid present had the D-configuration. This suggested that the easily liberated residue was D-aspartic acid. The second fraction containing aspartic acid was obtained by further hydrolysis of peptides that remained after the first aspartic acid had been removed. The behaviour of the second fraction to D-aspartic acid oxidase, which indicated that about 58% of the aspartic acid present had the L-configuration, favoured the view that the aspartic acid linked to lysine was derived from an L-aspartic acid residue. However, since the amount of racemization which appeared to have occurred was unexpectedly high, this view was thought to require confirmation. A second experiment was therefore carried out in which a more direct procedure was adopted.

In the second experiment, in which the time of the primary hydrolysis at 80° was reduced to 20 hr., the first aspartic acid to be liberated did not amount to more than one mole per mole of bacitracin. After isolation it was found to contain 93% of the D-isomer. A further sample of aspartic acid, isolated from peptides which had survived hydrolysis at 80° for 43 hr., was found to contain 76% of the L-isomer. These results leave no doubt that the easily liberated aspartic acid has the D-configuration and that the aspartic acid derived from the residue next to lysine has the L-configuration. The finding that the second aspartic acid had undergone more racemization than the first is consistent with the fact that more vigorous conditions are required for its liberation. When bacitracin is hydrolysed under conditions used for liberating both aspartic acid residues from the molecule, further racemization of the easily liberated aspartic acid is presumably responsible for the fact that the final product is almost completely racemic.

The peptide Asp.Lys gave a purple spot with the ninhydrin reagent used by LeQuesne & Young (1952), which has been reported to give a purple colour with α-aspartyl peptides and a blue colour with β-aspartyl peptides. If this test is reliable in the present case, the arrangement around the lysine residue in bacitracin A would now appear to be as shown in (II). An α-aspartyl linkage cannot




(II)

be accepted, however, without supporting evidence. Moreover, there are other properties of the peptides Asp.Lys and Asp(Ileu).Lys which are not yet understood and which raise the question whether they are more complicated than appears at first sight. Both these peptides behaved as though they had a small net negative charge during ionophoresis on paper at pH 7, although the structures assigned to them contain two free carboxyl groups and two free amino groups. When the DNP derivatives of Asp.Lys and Asp(Ileu).Lys were hydrolysed, the ether-soluble DNP-aspartic acid was accompanied by material which remained at the origin on paper chromatograms developed with the solvent system of Blackburn & Lowther (1951) and which appeared as a bright-green fluorescent spot when viewed under ultraviolet light. Similar material was obtained (together with DNP-isoleucine) by hydrolysing the DNP-derivative of bacitracin A, but its significance is at present uncertain. Weisiger, Hausmann & Craig (1955) also obtained a peptide containing aspartic and lysine from bacitracin A whose structure did not appear to be straightforward. The isolation from bacitracin A of the peptide Asp.Lys in amounts sufficient for more detailed study, and the synthesis of aspartyl-lysine peptides for comparative purposes, might help to throw more light on these questions.

## SUMMARY

1. When bacitracin A or deaminated bacitracin A was treated with 11N hydrochloric acid at 80° for 43 hr. most of the amino acids were liberated in the free state, but four peptides containing isoleucine or lysine resisted hydrolysis.

2. The surviving peptides were separated by ionophoresis and chromatography on paper and appeared to be Ileu.Phe, Ileu.Lys, Ileu(Asp)Lys, and Asp.Lys. The last was the most stable to further hydrolysis.

3. The DNP derivative of Ileu.Lys yielded DNP-isoleucine and ε-DNP-lysine on hydrolysis, and the DNP-derivative of Asp.Lys yielded DNP-aspartic acid and α-DNP-lysine.

4. It is concluded that an isoleucine residue is linked to the α-amino group of the lysine residue in bacitracin A and that an aspartic acid residue is

linked to the ε-amino group. However, certain properties of a substance containing lysine and aspartic acid raise the question whether its structure is more complicated than that of a simple dipeptide.

5. One of the aspartic acid residues in bacitracin A is readily liberated by hydrolysis in the free state. The second residue is linked to lysine by a bond which is unusually resistant to hydrolysis. A fraction which contained mainly the first aspartic acid was separated from a fraction which contained mainly the second. In one experiment the two fractions were subjected separately to the action of D-aspartic acid oxidase. In another the aspartic acid in each fraction was isolated and its specific rotation measured. The results indicate that the easily liberated aspartic acid is derived from a residue with the D-configuration and that the aspartic acid residue next to lysine has the L-configuration.

We wish to thank Dr F. Sanger, F.R.S., for a sample of α-DNP-lysine, and the Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A., for generous gifts of bacitracin. We are grateful to Miss M. Read and Mrs A. Talboys for expert technical assistance. One of us (I.M.L.) is indebted to the Medical Research Council for a Scholarship.

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# Isolation of Cephalosporin C, a Penicillin-like Antibiotic Containing D-α-Aminoadipic Acid

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(*Received* 26 *September* 1955)

Abraham, Newton & Hale (1954) described the purification of a hydrophilic antibiotic, cephalosporin N, which was produced by a species of *Cephalosporium* (I.M.I. 49137). Cephalosporin N yielded D-α-aminoadipic acid on hydrolysis, and the results of degradative experiments showed that it was probably (D-4-amino-4-carboxy-*n*-butyl)-penicillin (Newton & Abraham, 1954).

Crude preparations of cephalosporin N were reported to contain a second antibiotic named cephalosporin C (Newton & Abraham, 1955). The latter contained sulphur and gave D-α-aminoadipic acid on hydrolysis. Unlike cephalosporin N, it was stable in aqueous solution at pH 3 and showed an absorption band in ultraviolet light with a maximum at 260 mμ. This paper describes the isolation of cephalosporin C, in the form of a crystalline sodium salt, and gives some of its properties.

Cephalosporin C was first encountered as a distinct entity during the purification of crude cephalosporin N penillic acid by chromatography on an anion-exchange resin, when it followed the penillic acid from the column as a well-defined band. After isolation it was found to show

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anti-bacterial activity. Cephalosporin C was not formed from cephalosporin N during the conversion of the latter into its penillic acid at pH 3, because no cephalosporin C was present in the penillic acid prepared from highly purified cephalosporin N (Newton & Abraham, 1954). The possibility that it was formed at pH 3 from an impurity in crude cephalosporin N was later eliminated by isolating it without recourse to the penillic acid rearrangement. Many of the physical properties of cephalosporin C are very similar to those of cephalosporin N, and the separation of these two compounds proved more difficult than the separation of the former from cephalosporin N penillic acid. However, a partial resolution was achieved in a manner which left little doubt that cephalosporin C was present as such in the culture fluid of the *Cephalosporium*.

An account of some of the biological properties of cephalosporin C has been given by Florey (1955). The substance was remarkably non-toxic to the mouse, a dose of 100 mg. given intravenously having no noticeable ill effect. It was able to afford complete protection to mice infected with a strain of haemolytic streptococcus which killed untreated animals within 12 hr.

## RESULTS

Of the preparations of cephalosporin N examined, all except those that had been finally purified by countercurrent distribution (Abraham *et al.* 1954) were found to contain cephalosporin C. In the more active preparations, cephalosporin C was the main contaminant. It was largely responsible for the survival of some antibacterial activity when the cephalosporin N was converted into the corresponding penillic acid by keeping the preparations at pH 3 in aqueous solution. The amount of cephalosporin C in the preparations varied from batch to batch, but in most cases it was much smaller than the amount of cephalosporin N.

### *Separation of cephalosporin C from cephalosporin N penillic acid*

Cephalosporin C and cephalosporin N penillic acid were readily separated by chromatography on a column of Amberlite IR 4B or by countercurrent distribution in a phenol–carbon tetrachloride–water system containing acetic acid.

*Elution from Amberlite IR 4B.* Fig. 1 shows the elution curves obtained when two preparations of cephalosporin N penillic acid were chromatographed on a column of Amberlite IR 4B in ammonium acetate buffer, pH 5·0. The top curve was obtained with a preparation of the penillic acid from partly purified cephalosporin N (51 u./mg.). The material in the major peak was cephalosporin N penillic acid and that in the minor peak was cephalosporin C. The crystalline sodium salt of cephalosporin C was isolated from the contents of fractions 92–112. The bottom curve was obtained with a preparation of the penillic acid from a sample of cephalosporin N (68 u./mg.) that had been purified by countercurrent distribution in the manner described by Abraham *et al.* (1954).

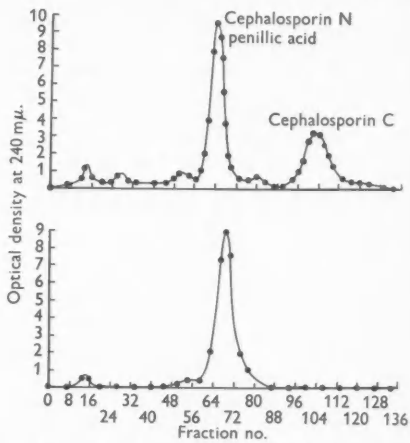
Cephalosporin N
penillic acid
Cephalosporin C
Optical density at 240 mμ.
Fraction no.
0 8 16 24 32 40 48 56 64 72 80 88 96 104 112 120 128 136

Fig. 1. Separation of cephalosporin C and cephalosporin N penillic acid on a column (1·7 cm. × 20 cm.) of Amberlite IR 4B. Elution with 0·2 M ammonium acetate, pH 5·0, 3 ml. fractions being collected. Top curve, crude penillic acid from partly purified cephalosporin N. Bottom curve, penillic acid from highly purified cephalosporin N.

Complete resolution of cephalosporin N penillic acid and cephalosporin C was also obtained on a column of Amberlite IR 4B when pyridine acetate, pH 5·0, was used as a buffer. This buffer was removed more easily than ammonium acetate from the products eluted from the column.

*Countercurrent distribution between solvents.* Fig. 2 shows the results obtained when a preparation of crude cephalosporin N penillic acid was subjected to ninety-seven transfers in a 100-tube machine, a solvent system composed of phenol, carbon tetrachloride and aqueous acetic acid being used. The distribution curve represents the ninhydrin colour densities given by samples from the upper (aqueous) layers. The material used in this experiment contained a considerable amount of substances other than cephalosporin N penillic acid and cephalosporin C. Cephalosporin N penillic acid was located in tubes 48–75 and cephalosporin C in tubes 20–50.

After the analysis shown in Fig. 2, the distribution was continued until 300 transfers had been completed. All the cephalosporin C was present in

fractions withdrawn from the machine between transfers 198 and 292. These fractions yielded crystalline cephalosporin C sodium salt.

*Separation of cephalosporin C from cephalosporin N*

*Elution from Amberlite IR 4B.* The recovery of active cephalosporin N after chromatography on a buffered ion-exchange resin was complicated by the instability of the substance at a pH lower than 5. Desalting by conventional methods, including those depending on the sublimation of volatile buffers, was unsuccessful. This difficulty was overcome by using pyridine sulphate as a buffer. Addition of the appropriate amount of barium hydroxide to a solution of either cephalosporin N or cephalosporin C in the buffer removed the sulphate ions and left the barium salt of the antibiotic in aqueous pyridine; the pyridine could then be removed *in vacuo* without loss of antibacterial activity. When mixtures of cephalosporin N and cephalosporin C were obtained, the maximum amount of the latter in the product could be estimated by measuring the optical density at 260 m$\mu$.

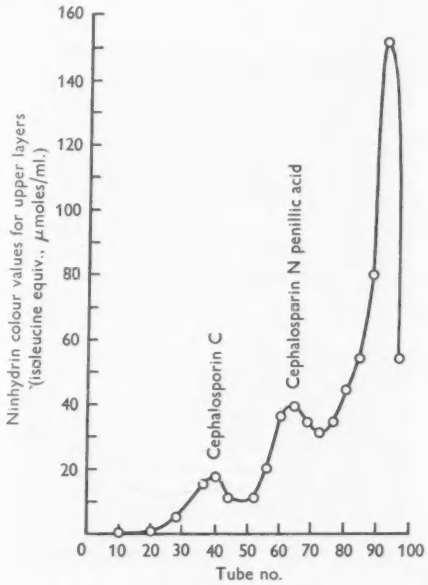
Ninhydrin colour values for upper layers (isoleucine equiv., μmoles/ml.)
Cephalosporin C
Cephalosparin N penillic acid
Tube no.

Fig. 2. Countercurrent distribution of crude cephalosporin N penillic acid in a phenol–carbon tetrachloride–water system containing acetic acid. The curve shows the fundamental series after ninety-seven transfers. The upper (aqueous) phase only was analysed.

Fig. 3 shows an elution curve obtained when a partly purified preparation of cephalosporin N (40 u./mg.) was chromatographed on a column of Amberlite IR 4B (20 cm. long) in pyridine sulphate buffer, pH 5·0, at 5°. The first small peak contained unidentified material without antibacterial activity. The major peak was asymmetrical. Material from its leading edge contained cephalosporin N virtually free from cephalosporin C. Material from its trailing edge contained a progressively large proportion of cephalosporin C and smaller proportion of cephalosporin N. Fractions 18–24 combined yielded a preparation of cephalosporin N barium salt with an activity of 50 u./mg. Fractions 30–45 yielded a crude barium salt of cephalosporin C still mixed with some cephalosporin N. This product was converted into its sodium salt. On concentrating an aqueous solution of the latter to a syrup the sodium salt of cephalosporin C separated as a crystalline mass.

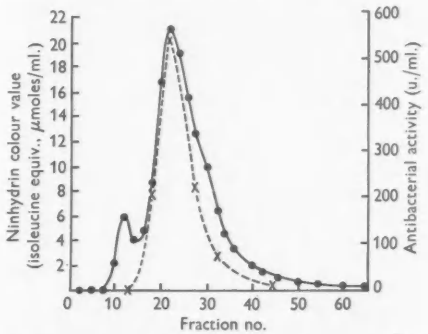


Fig. 3. Partial separation of cephalosporin C and cephalosporin N on a column (1·9 cm. × 20 cm.) of Amberlite IR 4B. Elution with pyridine sulphate, pH 5·0, 3 ml. fractions being collected. ●, Ninhydrin colour values; ×, antibacterial activity. Cephalosporin N is concentrated in the leading edge of the main peak, and cephalosporin C in the trailing edge.

In a similar experiment with a column 130 cm. long the trailing edge of the main peak was prolonged, but complete resolution of cephalosporin N and cephalosporin C was not obtained. This experiment yielded a preparation of the barium salt of cephalosporin N with an activity of 60 u./mg. and also a fraction from which cephalosporin C was obtained as a crystalline sodium salt.

*Separation on paper chromatograms.* On buffered paper chromatograms developed with methanol, which were used by Olson, Jennings & Junek (1953) for studying synnematin, cephalosporin N ran significantly faster than cephalosporin C. Thus

on paper buffered with sodium citrate, pH 5·5, cephalosporin N showed an $R_F$ value of 0·44 and cephalosporin C an $R_F$ value of 0·34. When a mixture of the two compounds was run on the chromatogram for 48 hr., the solvent being allowed to drip from the bottom of the paper, two clearly resolved spots were obtained on spraying with ninhydrin. Resolution that was nearly complete was obtained on paper buffered with sodium phosphate at pH 6·0. Fig. 4 shows the result of an experiment in which cephalosporin N (25 $\mu$g.), cephalosporin C (100 $\mu$g.) and a mixture of the two were run on a chromatogram under these conditions

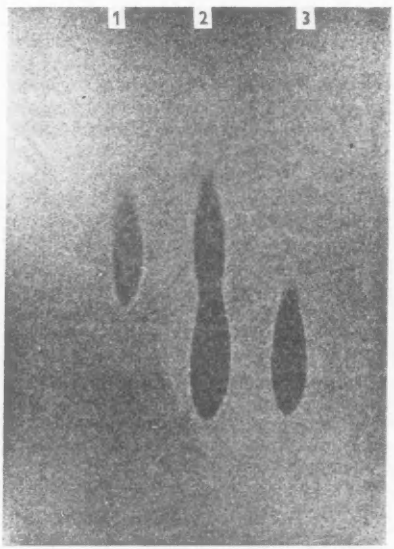


Fig. 4. Zones of inhibition on a plate seeded with *Salm. typhi*, showing the separation of cephalosporin C and cephalosporin N on Whatman no. 1 paper buffered with sodium phosphate, pH 6·0. The chromatogram was developed with methanol. 1, Cephalosporin C; 2, cephalosporin C and cephalosporin N; 3, cephalosporin N.

for 48 hr. at 4°. The paper was placed for 30 min. on a plate of agar seeded with a strain of *Salmonella typhi*, and the plate then incubated overnight. The positions of the antibiotics are marked by clear zones in an opaque area of bacterial growth.

Most of the crude preparations of cephalosporin N that were tested on paper chromatograms gave a spot in the position of cephalosporin C, as well as one in the position of cephalosporin N, when the paper was sprayed with ninhydrin. With the preparations that were richest in cephalosporin C the latter could also be distinguished from cephalosporin N on the chromatograms by the distribution of antibacterial activity. However, the fact that the activity/mg. of cephalosporin C was only about one-tenth of that of cephalosporin N made this impossible with other preparations in which cephalosporin C was present in lower concentration.

When subjected to ionophoresis on paper in collidine–acetate buffer, pH 7, in the manner described by Newton & Abraham (1954), cephalosporin C migrated towards the anode at almost the same rate as cephalosporin N. No separation of the compounds could be obtained under these conditions.

Fig. 5. Photomicrograph (×132) of cephalosporin C sodium salt, crystallized from aqueous *n*-propanol. The crystals were photographed between crossed nicol prisms.

*Some properties of cephalosporin C*

When preparations of the sodium salt of cephalosporin C were concentrated, in aqueous solution, to a syrup, crystallization began readily and continued until the preparation contained a solid mass of monoclinic crystals. Fig. 5 shows a product obtained by recrystallization from aqueous *n*-propanol. An X-ray crystallographic analysis was kindly carried out by Dr Dorothy Hodgkin and Mrs D. Mackay, who reported as follows. Unit cell dimensions: $a = 13{\cdot}12$ Å; $b = 4{\cdot}97$ Å; $c = 17{\cdot}82$ Å; $\beta = 106°$, 30. Space group, $P2_1$; number of molecules in the unit cell, 2; density, 1·38. The molecular weight calculated from these figures is $470 \pm 15$.

On drying *in vacuo* at room temperature crystalline cephalosporin C sodium salt, $[\alpha]_D^{20} + 103°$, lost 7·6% of its weight. This weight was rapidly regained on exposure to the laboratory air. The results of elementary analysis indicated that a possible formula for the hydrated product was $C_{16}H_{20}O_8N_3SNa,2H_2O$. Analytical figures for the amorphous free acid, obtained from the sodium salt by ionophoresis in a four-compartment cell (Synge, 1951), were consistent with this formula.

Cephalosporin C gave a positive ninhydrin reaction and a negative nitroprusside reaction. Electrometric titration indicated that it contained two acidic groups, with p$K$ values of 3·1 and $< 2·6$ respectively, and a basic group with a p$K$ value of 9·8. The equivalent weight, calculated from the span of the titration curve for the basic group, was $480 \pm 15$. In these properties cephalosporin C behaved as a monoaminodicarboxylic acid closely resembling cephalosporin N.

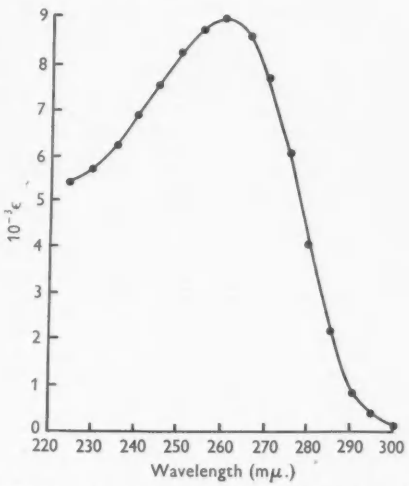


Fig. 6. Ultraviolet absorption spectrum of cephalosporin C sodium salt in water.

The ultraviolet absorption spectrum of cephalosporin C sodium salt in water ($\lambda_{max.}$ 260 mμ., $\epsilon_{max.}$ 9000) is shown in Fig. 6. The infrared spectrum showed bands at the following wavelengths: 2·94, 3·06, 5·61, 5·77, 6·05, 6·29, 6·57, 7·17 and 7·36 μ. The bands at 6·05 and 6·57 μ. could be attributed to the C=O of a monosubstituted amide, the band at 6·29 μ. to the carboxylate ion, the bands at 7·17 and 7·36 μ. to an *iso*propyl group, and the band at 5·77 μ. to an ester or lactone grouping. The band at 5·61 μ. is of particular interest because a band in this region is shown by cephalosporin N and the common penicillins, and has been attributed in the latter to the C=O of the β-lactam-thiazolidine ring system (Thompson, Brattain, Randall & Rasmussen, 1949).

When tested against a strain of *Salm. typhi* and against a strain of *Staphylococcus aureus* which did not produce penicillinase, cephalosporin C sodium salt showed an activity of 8–10 u./mg., the unit being that used by Abraham *et al.* (1954) for assaying cephalosporin N. Against these organisms cephalosporin C was only about one-tenth as active as cephalosporin N, but the two compounds showed similar activities against a strain of *Escherichia coli*.

Cephalosporin C was relatively stable to acid, losing no detectable activity on standing in 0·1 N-hydrochloric acid at room temperature for 4 hr. It was also more stable than cephalosporin N in the presence of heavy-metal ions, such as those of copper, zinc and mercury. It was rapidly inactivated, however, in alkaline solution above pH 11. Unlike cephalosporin N, it rapidly gave a heavy black precipitate when treated with sodium plumbite in 50% (w/w) sodium hydroxide solution at room temperature.

The sensitivity of cephalosporin C to four different preparations of penicillinase was compared with that of cephalosporin N under similar conditions. Table 1 shows the amount of each enzyme preparation required to destroy half the activity of a solution of cephalosporin C, relative to the amount required to destroy half the activity of a solution containing the same weight

Table 1. *Relative amounts of different preparations of penicillinase required to inactivate equal weights of cephalosporin C and cephalosporin N under similar conditions*

For details see Experimental section.

| Penicillinase | | Relative amounts required for 50% inactivation (amount for cephalosporin C/amount for cephalosporin N) |
|---|---|---|
| Source | Nature | |
| *B. subtilis* strain 749 | Crude | > 100 |
| *B. cereus* (NRRL 569) | Crude | 5 |
| *B. cereus* (NRRL 569) | Purified | 30 |
| *B. cereus* 5/B | Crystalline | 10 000 |

of cephalosporin N. A crude preparation of penicillinase from a strain of *Bacillus subtilis* had no effect on cephalosporin C in the highest concentration at which it was tested. A crude preparation of penicillinase from a strain of *Bacillus cereus* was active against cephalosporin C, but considerably less active than it was against cephalosporin N. The relative activity against cephalosporin C of a purified preparation of penicillinase from the same source was smaller still, and that of crystalline penicillinase from another strain of *B. cereus* (Pollock, Torriani & Tridgell, 1956) was very small indeed. These results are most easily explained by the assumption that the major component, at least, of the penicillinase produced by *B. subtilis* and *B. cereus* has no significant activity against cephalosporin C, but that the last-named is inactivated by a separate enzyme, C-cephalosporinase. However, they leave open the question whether this cephalosporinase has any activity against the penicillins.

## EXPERIMENTAL

### *General methods*

Antibacterial activity was assayed by the method used by Abraham *et al.* (1954), with *Salm. typhi* as the test organism and cephalosporin N as a standard. Photometric ninhydrin determinations were made by the method of Moore & Stein (1948). Electrometric titrations were carried out at 20° in a manner described by Newton & Abraham (1953). Infrared spectra of samples in paraffin paste were kindly measured by Dr F. B. Strauss, using a Perkin–Elmer double-beam photometer, model 21.

### *Paper chromatography*

Paper chromatograms were run on Whatman no. 1 paper which had been treated with 0·05 M sodium citrate buffer, pH 5·5, or with M sodium phosphate buffer, pH 6. Before use the paper was dipped in the buffer solution and dried in air at 37°. Usually about 100 $\mu$g. of material was applied to the paper. The chromatograms were developed with methanol which had been dried over CaO and distilled.

### *Chromatography on Amberlite resin*

*Resin.* The weakly basic anion-exchange resin Amberlite IR 4B (150–300 mesh) was used in these experiments. In each case the resin was first brought to equilibrium with the buffer solution used for elution (cf. Hirs, Moore & Stein, 1952).

*Buffer solutions.* The following buffers were used: (1) ammonium acetate, pH 5·0 (0·2 M), prepared as described by Hirs *et al.* (1952); (2) pyridine acetate, prepared by adding N pyridine (about 250 ml.) to 2N acetic acid (150 ml.) until the pH rose to 5·0, and diluting the solution to 1 l. with water; (3) pyridine sulphate, prepared by adding N pyridine (about 450 ml.) to 150 ml. of 2N-$H_2SO_4$ until the pH rose to 5·0 and diluting the solution to 1 l. with water.

*Separation of cephalosporin C from crude cephalosporin N penillic acid.* (*a*) Crude cephalosporin N penillic acid (98 mg.) was dissolved in 5 ml. of ammonium acetate buffer and the solution added to a column (1·7 cm. × 20 cm.) of Amberlite resin. Elution was carried out with ammonium acetate buffer, the flow rate being adjusted so that 3 ml. fractions were collected every 15 min. Samples taken from odd-numbered fractions were diluted 10 times with water and the optical densities of the resulting solutions were measured at 240 m$\mu$. (Fig. 1). Fractions 92–112 were combined, concentrated to a syrup in a rotating evaporator (Craig, Gregory & Hausmann, 1950), and finally dried *in vacuo* in a desiccator containing $P_2O_5$ and KOH. The solid was dissolved in water (25 ml.) and the solution placed in the specimen compartment of a four-compartment cell (Synge, 1951), fitted with a cooling coil carrying aqueous ethanol at −5°. Ionophoresis was continued until the current had fallen to 25 mA at 500 V. Cephalosporin C was recovered from the acetic acid compartment of the cell by freeze-drying. The product, which still retained some acetic acid, was dissolved in water (0·5 ml.) and precipitated with acetone (10 ml.). The sticky precipitate was separated by centrifuging and stirred with dry acetone until it formed a powder (7·7 mg.). The powder was dissolved in water (2 ml.), the pH adjusted to 6·5 by the addition of 0·1N-NaOH (0·17 ml.), and the resulting solution concentrated to a syrup. Within a few hours the syrup changed to a mass of crystals.

(*b*) Cephalosporin C was isolated in a similar manner in an experiment in which pyridine acetate was used instead of ammonium acetate. In this case the elution curve was obtained by measuring ninhydrin colour densities, using 0·2 ml. samples of alternate fractions from the column. Ionophoresis in the four-compartment cell was unnecessary for the complete removal of the buffer at room temperature. The fractions containing cephalosporin C were evaporated *in vacuo* and the product precipitated from a small volume of water by acetone. The precipitate was dissolved in water, the pH of the solution brought to 7·5 with NaOH, and the solution of the resulting sodium salt then concentrated *in vacuo*. On standing, the resulting syrup changed to a mass of crystals.

*Separation of cephalosporin C from cephalosporin N.* Partly purified cephalosporin N (600 mg., 41 u./mg.) was dissolved in 4 ml. of pyridine sulphate buffer and added to a column (1·9 cm. × 20 cm.) of Amberlite resin. The column was surrounded by a jacket carrying running water at 5°. Elution was carried out with pyridine sulphate buffer, 3 ml. fractions being collected every 20 min. Each fraction was collected in a tube containing 6 drops of pyridine, whereby its final pH was raised to 6. Photometric ninhydrin determinations were made on 0·1 ml. samples of alternate fractions, and samples from a few selected fractions were withdrawn for measurement of antibacterial activity (Fig. 3). Fractions 18–24 were combined and 0·3N-$Ba(OH)_2$ was added until the pH was 7·6. The precipitate was removed by centrifuging and the supernatant freeze-dried to yield a preparation of the barium salt of cephalosporin N (195 mg.) as a white powder. Its activity was 50 u./mg. Traces of pyridine still remaining in this product were removed by dissolving it in water (2 ml.), adjusting the pH to 7·2, and precipitating the product by the addition of 40 ml. of acetone.

Fractions 30–45 were combined and treated in a similar manner. They yielded 44 mg. of a crude barium salt of cephalosporin C. This product was dissolved in water and

a solution of $Na_2SO_4$ (10 mg./ml.) added in an amount just sufficient to precipitate the $Ba^{2+}$ as $BaSO_4$. After centrifuging, the supernatant solution, containing the sodium salt of cephalosporin C, was concentrated *in vacuo* to a syrup. On standing the latter changed to a crystalline mass.

### *Countercurrent distribution*

*Solvent system.* This was made by mixing phenol saturated with water (2 l.), $CCl_4$ (0·8 l.), water saturated with phenol (9 l.), and glacial acetic acid (0·2 l.).

*Separation of cephalosporin C from crude cephalosporin N penillic acid.* Crude cephalosporin N penillic acid (40 g.) was dissolved in 75 ml. of the upper layer and 60 ml. of the lower layer of the solvent system. The pH of the upper layer was adjusted to 3·1 with 11 N-HCl (6·5 ml.). The solutions were then added to tubes 0, 1 and 2 of an all-glass, automatic, countercurrent-distribution machine, the fixed vol. of the lower layer in each tube being 20 ml. and the vol. of the mobile upper layer 27 ml. The machine was first set to complete 97 transfers, by means of the fundamental procedure (Craig & Craig, 1950). Samples (4·8 $\mu$l.) from the upper layer of every fourth tube were analysed by the photometric ninhydrin procedure (Fig. 2). The machine was then set to carry out a further 200 transfers, the upper layers being withdrawn from tube 100. The fractions withdrawn during transfers 99–164 were not examined further. Photometric ninhydrin determinations were made on samples (20 $\mu$l.) of alternate fractions withdrawn during transfers 165–300. Antibacterial activities were measured on samples (1·0 ml.) of every sixth fraction withdrawn during transfers 182–296. For this purpose the samples were diluted with 1 vol. of 0·2 M-$Na_2POH_4$ and the resulting solutions extracted twice with 1 vol. of $CCl_4$ to remove phenol. The final pH of the solutions was 6·8. The ninhydrin and activity determinations indicated that cephalosporin C was located in the fractions withdrawn during transfers 198–292.

*Recovery of cephalosporin C.* The pooled withdrawn fractions containing cephalosporin C were evaporated to about 250 ml. in a rotating evaporator (Craig *et al.* 1950) and the concentrated solution was extracted first with 2 vol. of $CCl_4$ and then three times with 1 vol. of benzene. The pH of the aqueous solution was then adjusted to 3·3 by the addition of pyridine, the solution concentrated to 50 ml. in the rotating evaporator and the concentrate freeze-dried. The resulting cephalosporin C was dissolved in a small volume of water and precipitated by the addition of 20 vol. of acetone. The precipitate was washed with acetone and dissolved in water, and the pH of the solution brought to 7·5 by addition of 2 N-NaOH. On concentrating the solution to a syrup, cephalosporin C separated as the crystalline sodium salt. The total yield was 800 mg.

### *Isolation and properties of cephalosporin C*

Concentration of solutions of the sodium salt of cephalosporin C gave a syrup which crystallized on standing. The crystalline mass was stirred with a small amount of 70% (v/v) ethanol, which dissolved amorphous material, and the crystals were removed by filtration. The product was recrystallized by dissolving it in a small volume of water, slowly adding *n*-propanol until crystallization began, and then allowing the mixture to stand in the cold room. The air-dried crystalline sodium salt had $[\alpha]_D^{20} + 103°$ in water (*c*, 0·9). When dried *in vacuo* at room temperature (or at 40°) it lost weight (7·5%) which was rapidly regained on exposure to the laboratory air. Elementary analyses were made on the air-dried products. (Found: C, 40·0; H, 5·2; N, 8·9; S, 6·2; Na, 4·9. Calc. for $C_{16}H_{20}O_8N_3SNa,2H_2O$: C, 40·5; H, 5·1; N, 8·9; S, 6·7; Na, 4·9; $2H_2O$, 7·5%; mol.wt. 473.)

Amorphous cephalosporin C free acid was prepared by ionophoresis of the crystalline sodium salt in the four-compartment cell. The free acid, obtained by freeze-drying the contents of the acetic acid compartment, was precipitated from aqueous solution by addition of 20 vol. of acetone. The air-dried product lost weight (4·2%) on drying *in vacuo* at room temperature. Elementary analyses were made on the air-dried product. (Found: C, 44·4; H, 5·6; N, 9·8; S, 7·3. Calc. for $C_{16}H_{21}O_8N_3S,H_2O$: C, 44·5; H, 5·3; N, 9·7; S, 7·4%.)

*Stability to heavy-metal ions.* Solutions containing 0·2 mg. of cephalosporin C sodium salt and 1 mg. of $CuSO_4,5H_2O$, $Pb(NO_3)_2$ or $ZnSO_4$ in 1 ml. of water were kept at 37° for 3 hr. Antibacterial assays showed that no detectable activity had been lost in the presence of $Cu^{2+}$ and $Zn^{2+}$ and that the loss was less than 50% in the presence of $Pb^{2+}$. Under similar conditions cephalosporin N was completely inactivated.

*Effect of penicillinase preparations.* Four different preparations of penicillinase were used: (1) a solution of crude penicillinase from *B. subtilis* strain 749 (cf. Manson, Pollock & Tridgell, 1954), kindly provided by The Distillers Co. (Biochemicals) Ltd., (2) a crude freeze-dried preparation of penicillinase from *B. cereus* strain 569; (3) a highly purified preparation of penicillinase from *B. cereus* strain 569 (Pollock & Torriani, 1953); (4) twice-crystallized penicillinase from *B. cereus* strain 5/B, a constitutive mutant from a strain of *B. cereus* which does not produce penicillinase (Pollock *et al.* 1956). The three preparations of penicillinase from *B. cereus* were kindly provided by Dr M. R. Pollock.

Solutions of cephalosporin C sodium salt (1 mg.) in 0·9 ml. of 0·6 M sodium phosphate buffer, pH 7·0, were mixed with 0·1 ml. samples of solutions of the penicillinase and the mixtures kept at 37° for 3 hr. Each preparation of penicillinase was used in a series of decreasing concentrations, made by serial dilution of a stock solution of appropriate strength. Solutions of cephalosporin N (1 mg.) were treated in the same way as those of cephalosporin C. After incubation, the solutions containing penicillinase and control solutions to which no enzyme had been added were assayed against *Salm. typhi.* Under these conditions the degree of inactivation was normally reduced from more than 75% to less than 25% over a tenfold dilution of the enzyme solution. The dilution at which about 50% inactivation occurred was taken as the end point.

This method of measuring enzyme activities was a relatively crude one, since enzymic action continued during the period of assay. However, the method was simple and the results were sufficiently clear-cut to justify its use in the present case.

## SUMMARY

1. Crude preparations of cephalosporin N, a hydrophilic penicillin, have been found to contain a second hydrophilic antibiotic. This substance, named cephalosporin C, has been isolated as a crystalline sodium salt.

2. Mixtures of cephalosporin C and cephalosporin N penillic acid have been completely resolved by chromatography on an anion-exchange resin and by countercurrent distribution in a phenol–water system containing acetic acid. Mixtures of cephalosporin C and cephalosporin N have been resolved on paper chromatograms and partly resolved on an ion-exchange resin.

3. Cephalosporin C has been provisionally assigned the molecular formula $C_{16}H_{21}O_8N_3S$. It behaves as a monoaminodicarboxylic acid. It shows an absorption maximum at 260 m$\mu$. and its infrared spectrum contains a band at 5·61 $\mu$.

4. Cephalosporin C shows similar activity to *Salm. typhi* and *Staph. aureus*. Against these organisms its activity is about 10% of that of cephalosporin N.

5. Cephalosporin C is stable in dilute acid but readily inactivated by dilute alkali. It is insensitive to some preparations of penicillinase, but not to others. A new enzyme, C-cephalosporinase, may therefore catalyse its inactivation.

We are grateful to the staff of the Medical Research Council's Antibotic Research Station, Clevedon, for supplies of crude cephalosporin N and to Dr M. R. Pollock and The Distillers Co. (Biochemicals Ltd.) for gifts of penicillinase. We wish to thank Miss M. Read and Mr O. Boys for expert technical assistance.

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# Experiments on the Degradation of Cephalosporin C

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(*Received* 26 *September* 1955)

The isolation and some of the properties of cephalosporin C have been described by Newton & Abraham (1956). This substance behaves as a monoaminodicarboxylic acid and has been given a provisional molecular formula which differs from that assigned to cephalosporin N (D-4-amino-4-carboxy-*n*-butylpenicillin) by only two carbon and two oxygen atoms. Moreover, the infrared spectrum of the substance shows a band at 5·61 $\mu$. which is believed to be characteristic of the penicillins. These facts indicate that cephalosporin C is related to cephalosporin N, but other properties of cephalosporin C, such as its relative stability to acid and to crystalline penicillinase, suggest that it should not be regarded as a true penicillin. This paper gives an account of some of the changes which occur on hydrolysis of cephalosporin C and describes the isolation, from both cephalosporin C and the material formed from it by hydrogenolysis with Raney nickel, of degradation products showing a positive ninhydrin reaction.

Electrometric titration of cephalosporin C with 0·1N sodium hydroxide revealed the presence of a group with a p$K$ of 9·8. At a pH above 11 the substance underwent rapid hydrolysis, with loss of activity and of the absorption maximum at 260 m$\mu$. (Fig. 1). After standing at pH 12 for 2 hr., back titration showed that two new acidic groups had been formed and that buffering now occurred in the pH range of 4–6 (Fig. 2). The resulting solution gave a positive nitroprusside test, but the titration revealed no new group with a p$K$ that seemed likely to be that of a simple thiol. The characteristic absorption band of cephalosporin C at 260 m$\mu$. had disappeared.

* Member of the Scientific Staff, Medical Research Council.

When alkali-inactivated cephalosporin C was subjected to ionophoresis on paper in collidine–acetate buffer, pH 7 (Newton & Abraham, 1954), and the paper sprayed with ninhydrin, it showed a purple spot due to neutral material together with a blurred and rather complicated pattern due to material which had migrated towards the anode. This picture was simplified by more vigorous alkaline hydrolysis with 0·3 N barium hydroxide at 100° for 2 hr. Ionophoresis then showed a neutral spot with the reddish tint typical of glycine (Woiwod, 1949) and a single acidic spot corresponding to α-aminoadipic acid. After chromatography on paper there were two major spots in the positions of glycine and α-aminoadipic acid respectively. No additional spot was obtained when the material was treated with bromine water before application to the paper.

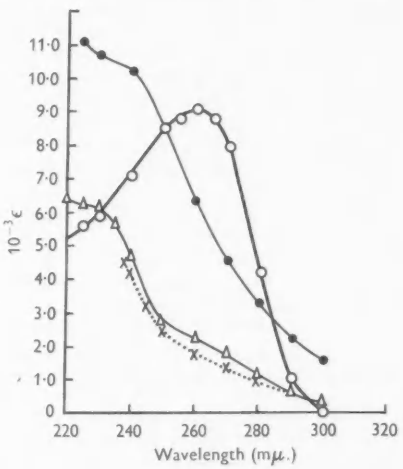



Fig. 1. Ultraviolet absorption spectrum of cephalosporin C and of products formed from it on hydrolysis. —○—, Cephalosporin C sodium salt; —●—, after treatment with 0·1 N-HCl at 100° for 1 hr.; · · × · ·, after treatment with 0·1 N-$Ba(OH)_2$ at 20° for 1 hr.; —△—, after treatment with C-cephalosporinase.

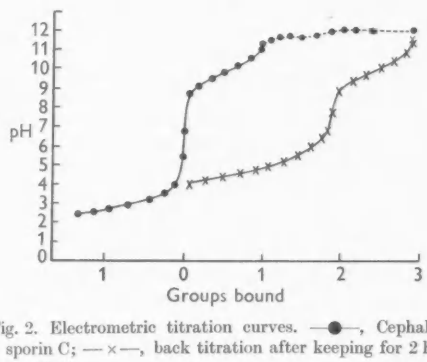



Fig. 2. Electrometric titration curves. —●—, Cephalosporin C; — × —, back titration after keeping for 2 hr. at pH 12.

The reaction which occurred when cephalosporin C was treated with a preparation of penicillinase that contained a cephalosporinase (Newton & Abraham, 1956) resulted in the liberation of acid. The reaction at 30° was followed by allowing sodium hydroxide to run into the stirred solution at such a rate that the pH, measured by a glass electrode, remained in the vicinity of 7·0. After 80 min., when nearly one acid group was liberated, the reaction had become very slow and the experiment was terminated. Measurement of the ultraviolet absorption spectrum of the resulting solution showed that the absorption band at 260 mμ. had almost entirely disappeared (Fig. 1).

Cephalosporin C could be kept in 0·1 N hydrochloric acid for 4 hr. without any detectable change in its ultraviolet absorption spectrum, but in 0·1 N hydrochloric acid at 100° the absorption maximum at 260 mμ. rapidly disappeared (Fig. 1). In a similar experiment with 0·1 N sulphuric acid nearly 1 mole of carbon dioxide was evolved in 1 hr. On treatment with N hydrochloric acid at 105° for 8 hr. 1·8 equivalents of volatile base were liberated. When subjected to chromatography or ionophoresis on paper, the hydrolysate showed a major ninhydrin-positive spot, in a position corresponding to that of α-aminoadipic acid and a faint spot in a position corresponding to glycine. This picture was virtually unchanged when the hydrolysate was treated with bromine water before application to the paper. Fig. 3 shows the results obtained when acid hydrolysates of cephalosporin C, cephalosporin N penillic acid, and a mixture of benzylpenicillin and α-aminoadipic acid were each treated with bromine water and chromatographed on paper in butanol–acetic acid. The hydrolysate of cephalosporin C differs sharply from the other two in giving no spot corresponding to penicillaminic acid (β-sulphovaline). Ionophoresis in a four-compartment cell (Synge, 1951) of the product of hydrolysis of cephalosporin C yielded an acidic fraction from which D-α-aminoadipic acid ($[\alpha]_D^{20} - 25°$ in 6 N-HCl; m.p. 205° and mixed m.p. 205–208°) was isolated in crystalline form.

Cephalosporin C was allowed to react with 1-fluoro-2:4-dinitrobenzene in aqueous sodium bicarbonate, and after adjustment of the pH of the mixture to 3·0 the product was extracted with ethyl acetate. Antibacterial assay of the ethyl acetate-soluble material showed that it was more active against *Staphylococcus aureus* and considerably less active against *Salmonella typhi* than

cephalosporin C. This material was hydrolysed with 6N hydrochloric acid at 105° for 10 hr. The resulting ether-soluble dinitrophenyl (DNP) derivative behaved like DNP-α-aminoadipic acid on paper chromatograms run under the conditions described by Blackburn & Lowther (1951).

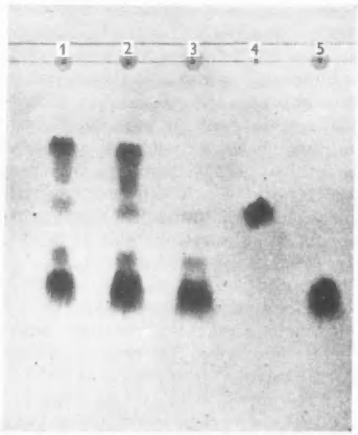



Fig. 3. Paper chromatogram of acid hydrolysates of cephalosporin C and other substances after treatment with bromine water. 1, Benzylpenicillin plus α-aminoadipic acid; 2, cephalosporin N penillic acid; 3, cephalosporin C; 4, glycine; 5, α-aminoadipic acid. The top spot in 1 and 2 is penicillaminic acid.

The results of these experiments showed that cephalosporin C contained a residue of D-α-aminoadipic acid whose amino group was free. The presence in cephalosporin C of a group with a p$K$ of 9·8, a value very close to that of the amino group of free α-aminoadipic acid, suggested that the α-carboxyl group of the α-aminoadipic acid residue was also free. It appeared probable, therefore, that this residue was joined to the rest of the molecule through its δ-carboxyl group.

Treatment of cephalosporin C with benzoyl chloride in sodium bicarbonate solution yielded a benzoyl derivative which was extracted into *n*-butanol at pH 2 and isolated as an amorphous sodium salt. This product showed an activity against *Staph. aureus* which was at least twice that of cephalosporin C, but an activity against *Salm. typhi* which was only one-quarter of that of cephalosporin C. A similar change in activity was noted on benzoylation of cephalosporin N (Newton & Abraham, 1954).

Hydrogenation of cephalosporin C in a Warburg respirometer, with palladium-on-charcoal as a catalyst, resulted in the uptake of nearly 1 mole of hydrogen in 2·5 hr. The amorphous product still showed an absorption maximum at 260 mμ. but it had only about 10% of the antibacterial activity of cephalosporin C. Hydrolysis of this product with N hydrochloric acid at 105° gave material which showed two ninhydrin-positive spots on a two-dimensional paper chromatogram run first with butanol–acetic acid and then with phenol. One spot corresponded with α-aminoadipic acid and the other with glycine. The latter spot was less intense than the former, but was relatively stronger than the glycine spot obtained from cephalosporin C which had not been hydrogenated before hydrolysis with acid. No visible change occurred in the spots corresponding to α-aminoadipic acid and glycine when the hydrolysate of the product of hydrogenation was treated with bromine water before application to a chromatogram run with butanol–acetic acid. In this case, however, a weak new spot appeared in the position of penicillaminic acid (β-sulphovaline).

Hydrogenolysis of cephalosporin C with Raney nickel yielded material which showed a complicated picture on paper chromatograms run with butanol–acetic acid. This picture was simplified considerably when the material was hydrolysed with N hydrochloric acid at 105°. The chromatogram then showed two main ninhydrin-positive spots in positions corresponding to α-aminoadipic acid and valine respectively, and two weaker spots. One of the last corresponded to glycine and the other, not yet identified, had an $R_F$ value of about 0·13. The major portion of the material that was found in the position of α-aminoadipic acid on the chromatogram migrated towards the anode at the same rate as α-aminoadipic acid during ionophoresis at pH 7. However, the ionophoresis revealed that a small amount of neutral substance was present in this material. The fact that the latter had shown an $R_F$ value similar to that of α-aminoadipic acid when chromatographed in butanol–acetic acid suggested that it might be alanine.

In a later experiment an acid hydrolysate of the products of hydrogenolysis of cephalosporin C was prepared on a larger scale. The results which had been obtained by paper chromatography and ionophoresis were confirmed and extended by the isolation from this hydrolysate of three amino acids. The material was first separated into neutral and acidic fractions by ionophoresis in a four-compartment cell. The neutral fraction was chromatographed on a column of Dowex 50-X4, elution being effected with N hydrochloric acid (cf. Hirs, Moore & Stein, 1954). Analysis of the effluent from the column by the photometric ninhydrin procedure (Moore & Stein, 1954) gave three main peaks as shown in Fig. 4. Fractions 46–51, 55–58

and 67–79 were pooled, giving combined fractions labelled *A*, *B* and *C* respectively. On evaporation to dryness all three fractions yielded crystalline residues. On paper chromatograms run with butanol–acetic acid or with phenol, *A* was indistinguishable from glycine, *B* from alanine and *C* from valine. In 6N hydrochloric acid, *B* showed a positive rotation ($[\alpha]_D^{20}$ approximately $+8°$) and *C* a small negative rotation ($[\alpha]_D^{20}$ approximately $-5°$).

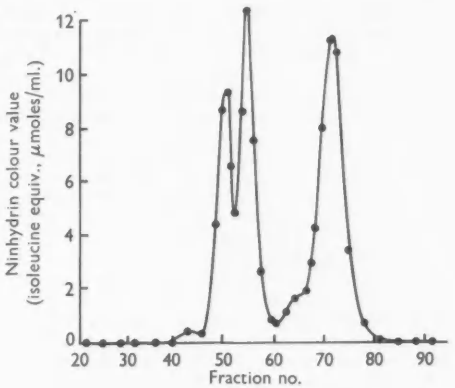



Fig. 4. Chromatography on Dowex 50-X4 of neutral material formed on acid hydrolysis of the product of hydrogenolysis of cephalosporin C. The column was 0·9 cm. × 41 cm. Elution was with N-HCl and 1·5 ml. fractions were collected.

After passage through a short column of Dowex 1 acetate to remove any remaining hydrochloric acid (Hirs *et al.* 1954), the three substances were crystallized from aqueous ethanol. The products were identified as glycine, alanine and valine respectively by comparisons of their infrared spectra with those of authentic samples of these amino acids. The valine was also characterized by elementary analysis.

The acidic fraction from the four-compartment cell was chromatographed on a column of Dowex 1 acetate, elution being carried out with 0·5N acetic acid as described by Hirs *et al.* (1954). The effluent curve, obtained from determinations of dry weight, is shown in Fig. 5. A white crystalline product separated from fractions 19, 20 and 21 within a few hours of their collection. Elementary analysis and other measurements ($[\alpha]_D^{20} - 25°$ in 6N-HCl; m.p. and mixed m.p. 207–208°) identified this substance as D-α-aminoadipic acid. Fractions 11–12 and 129–133 yielded partly crystalline residues, on evaporation to dryness, which have not yet been characterized.

The work described here provides only a limited amount of information about the structure of cephalosporin C, but it shows clearly some of the chemical similarities and dissimilarities of this substance to cephalosporin N and other members of the penicillin family. In containing a residue of D-α-aminoadipic acid linked to the rest of the molecule through its δ-carboxyl group, cephalosporin C resembles cephalosporin N, and in yielding carbon dioxide and traces of glycine on hydrolysis

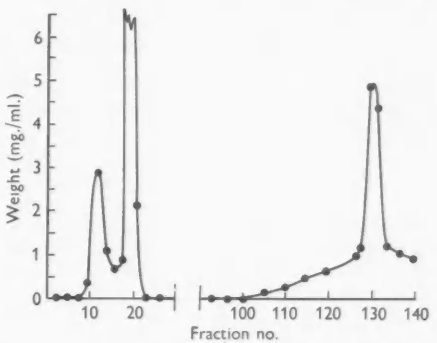
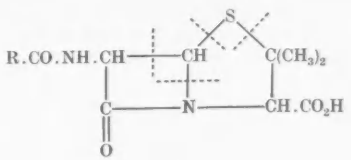



Fig. 5. Chromatography on Dowex 1 × 10 acetate of acidic material formed on acid hydrolysis of the product of hydrogenolysis of cephalosporin C. The column was 0·91 cm. × 36 cm. Elution was with 0·5N acetic acid and 1·0 ml. fractions were collected. No material was eluted in fractions 30–90.

with acid it resembles cephalosporin N and benzylpenicillin. The glycine from the latter compounds presumably arises by oxidation of the potential aminoacetaldehyde fragment of the penicillin molecule. The material obtained by hydrogenolysis of cephalosporin C resembles the corresponding material from benzylpenicillin in yielding valine, alanine and glycine on hydrolysis. The formation of these amino acids from benzylpenicillin involves the breaking of C–N and C–C bonds as shown in (I).




(I)

The alanine from benzylpenicillin (Kaczka & Folkers, 1949), like that from cephalosporin C, has largely the L-configuration. The valine from benzylpenicillin has the D-configuration, but that obtained

from cephalosporin C, while having an excess of the D- over the L-form, appeared to be extensively racemized.

Cephalosporin C appears to yield no hydrogen sulphide on hydrolysis at 105° with N hydrochloric acid, and very little on hydrolysis with 0·3N barium hydroxide, but its reaction with strongly alkaline lead plumbite (Newton & Abraham, 1956) indicates that it contains a labile sulphur atom. However, the failure of this antibiotic to yield a detectable amount of penicillamine ($\beta$-thiolvaline) on hydrolysis, or of penicillaminic acid ($\beta$-sulphovaline) on hydrolysis followed by treatment with bromine water, distinguishes it sharply from the normal penicillins. When considered in conjunction with the results of hydrogenation and hydrogenolysis, and with the fact that two equivalents of volatile base are liberated on hydrolysis under conditions in which only one equivalent is formed from cephalosporin N and benzylpenicillin, this raises the question whether cephalosporin C contains a penicillamine derivative with four substituents on the $\alpha$-carbon atom, one of which is an oxygen atom and is removed by reductive fission in the presence of Raney nickel.

## EXPERIMENTAL

Electrometric titrations were carried out in the manner described by Newton & Abraham (1953). Infrared spectra of samples in paraffin paste were kindly measured by Dr F. B. Strauss, using a Perkin–Elmer Double Beam photometer, model 21.

*Paper chromatography and ionophoresis.* Paper chromatograms were run on Whatman no. 1 paper, the butanol–acetic acid mixture of Woiwod (1949) or 80% (w/w) phenol being used in an atmosphere saturated with 50% (v/v) acetic acid (Dent, 1948). Ionophoresis on Whatman no. 1 paper was carried out in collidine acetate buffer (pH 7) in the manner described by Newton & Abraham (1954). In most cases about 100 $\mu$g. of material was applied to the paper.

### *Hydrolysis of cephalosporin C*

*Hydrolysates.* For chromatography and ionophoresis on paper hydrolysis at 105° was carried out in sealed tubes with about 2 mg. of cephalosporin C in 2 ml. of N-HCl or of $Ba(OH)_2$ (0·1 or 0·3N). The HCl was subsequently removed *in vacuo* and the $Ba^{2+}$ by precipitation with $H_2SO_4$. In some cases the products were treated with $Br_2$ water. This was done by passing $Br_2$ vapour into the solution, until no more was absorbed, and rapidly removing any excess *in vacuo*.

*Evolution of $CO_2$ on hydrolysis.* This was measured in the apparatus described by Abraham, Baker, Chain & Robinson (1949). Cephalosporin C sodium salt (20 mg.) was dissolved in 2·5 ml. of 0·1N-$H_2SO_4$ and 0·1 ml. of N-$H_2SO_4$ was added. The solution was refluxed in a stream of $CO_2$-free air which was then passed through 0·1N-$Ba(OH)_2$. A precipitate began to form in the $Ba(OH)_2$ solution within 3 min. After 1 hr., back titration with 0·1N-HCl indicated that 0·8 mole of $CO_2$ had been evolved. No further $CO_2$ was evolved on refluxing for an additional 30 min.

*Enzymic inactivation.* Cephalosporin C sodium salt (4·9 mg.) was dissolved in 1 ml. of water and a solution of partly purified penicillinase from *B. cereus* 569, kindly provided by Dr M. R. Pollock (1 ml., containing about 50 $\mu$g. of solid), was added. The solution was kept at 30° and stirred by a stream of $N_2$ while the pH, measured by a glass electrode, was maintained at 7 by the addition of 0·1N-NaOH from a micro-burette. The rate of reaction began to diminish after 40 min. and after 80 min., when it was very slow, the experiment was terminated. The vol. of 0·1N-NaOH added (0·98 ml.) then corresponded to 0·95 equiv. The product showed $E^{1\%}_{1\,cm.}$ 42 at 260 m$\mu$. when its absorption was measured relative to a blank containing the enzyme alone. The original material showed $E^{1\%}_{1\,cm.}$ 180 at 260 m$\mu$.

*Isolation of D-$\alpha$-aminoadipic acid from an acid hydrolysate of cephalosporin C.* Crystalline sodium salt of cephalosporin C (50 mg.) was hydrolysed with N-HCl (5 ml.) at 108° for 16 hr. under $N_2$ in a sealed tube. The hydrolysate was evaporated to dryness *in vacuo*, and then separated into acidic, basic and neutral fractions in a four-compartment cell (Synge, 1951). After the cell had been run for 4 hr. and the current had fallen to 25 mA at 500V the contents of the acetic acid compartment were evaporated to dryness in a rotary evaporator (Craig, Gregory & Hausmann, 1950). The solid was dissolved in 2 ml. of water, acetic acid (0·5 ml.) was added and the solution slowly evaporated *in vacuo* to about 1 ml. A crystalline product separated which was filtered off, washed with water and dried (3 mg.). It had $[\alpha]_D^{16} - 25°$ in 6N-HCl ($c$, 0·68); m.p. 205°. The mixed m.p. with an authentic sample of D-$\alpha$-aminoadipic acid (m.p. 207–208°, kindly supplied by Dr J. P. Greenstein) was 205–208°. The product behaved like $\alpha$-aminoadipic acid when subjected to ionophoresis on paper at pH 7 or to chromatography on paper in phenol or butanol–acetic acid.

*Volatile base formed on acid hydrolysis.* Samples (1 mg.) of cephalosporin C sodium salt, highly purified cephalosporin N barium salt, and sodium benzylpenicillin were hydrolysed with N-HCl at 108° for 16 hr. Each hydrolysate was washed into a Markham still (Markham, 1942) and the steam-volatile base liberated on the addition of 40% (w/v) NaOH was titrated. The amount of volatile base obtained from cephalosporin C was 1·8 equiv./mole. The amount obtained from both cephalosporin N and benzylpenicillin was 0·9 equiv./mole.

### *Derivatives of cephalosporin C*

*Reaction with 1-fluoro-2:4-dinitrobenzene (FDNB).* Cephalosporin C sodium salt (5·8 mg.) was added to a mixture of FDNB (6 mg.), ethanol (6 ml.), $NaHCO_3$ (4·3 mg.), and water (6 ml.). After 3 hr. at 20°, the optical density, at 350 m$\mu$., of a sample of the reaction mixture, diluted ten times, was 0·54 relative to a control solution from which the cephalosporin C was omitted. This value would be expected if 50% of one amino group in cephalosporin C had been substituted, assuming that the molecular extinction coefficient of DNP-cephalosporin C, like that of other DNP-substituted peptides (Battersby & Craig, 1952), is 14 500. The reaction was then stopped by neutralizing the solution with dilute acid and evaporating the ethanol *in vacuo*. The neutral aqueous residue was extracted with ether to remove unchanged FDNB. Samples of the re-

sulting solution assayed at 2·6 u./ml. against *Salm. typhi* and 12 u./ml. against *Staph. aureus*. A sample of the starting mixture from which FDNB had been omitted assayed at 8 u./ml. against both test organisms.

The remainder of the solution (4 ml.) was acidified to pH 3·0, extracted twice with ethyl acetate (4 ml.), and the ethyl acetate washed with 0·01 N-HCl (1 ml.). The ethyl acetate was then shaken with 2 ml. of water and 0·1 N-NaOH was added until the pH of the aqueous phase at equilibrium was 6·0. Ether (12 ml.) was added to the mixture and the aqueous phase was evaporated to dryness, yielding 5·4 mg. of material. The crude product was probably contaminated with sodium acetate derived from acetic acid present in the ethyl acetate. Its antibacterial activity was 18 u./mg. against *Staph. aureus* and about 1 u./mg. against *Salm. typhi*.

*Hydrolysis of DNP-cephalosporin C.* Crude DNP-cephalosporin C (4·5 mg.) was hydrolysed with 6 N-HCl under $N_2$ for 10 hr. at 105°. The hydrolysate was extracted with ether and the extract yielded 0·6 mg. of a yellow solid on evaporation. The products of hydrolysis were examined by ionophoresis and chromatography on paper. The water-soluble fraction was found to contain little, if any, α-aminoadipic acid. The ether-soluble fraction gave a major spot which behaved like DNP-α-aminoadipic acid when examined by paper ionophoresis in collidine acetate buffer, pH 7, and by paper chromatography in the system of Blackburn & Lowther (1951). By comparison with known amounts of authentic DNP-α-aminoadipic acid, the amount of DNP-α-aminoadipic acid in 100 μg. of the ether-soluble fraction was estimated to be about 25 μg.

*Benzoylation.* Cephalosporin C sodium salt (50 mg.; 8 u./mg.) was dissolved in 3% (w/v) $NaHCO_3$ solution and the pH brought to 8·5 with 2 drops of 0·1 N-NaOH. Acetone (1 ml.) was added and the solution cooled in ice and stirred, while benzoyl chloride (16 mg.) in acetone (2 ml.) was added slowly during 5 min. After stirring for 20 min., the ice-bath was removed and stirring continued for a further 10 min. The pH of the solution was then adjusted to 6·5 with N-HCl and the acetone removed *in vacuo*. The resulting solution was acidified with N-HCl. A precipitate began to form when the pH fell to 3·6 and reached a maximum at about pH 2. The mixture was then shaken twice with 0·5 vol. of *n*-butanol. Evaporation of the wet butanol extract *in vacuo* left an amorphous solid (37·3 mg.). The latter was dissolved in ethanol (1 ml.) and after addition of water (1 ml.) the pH of the solution was adjusted to 7·0 with 0·1 N-NaOH. Evaporation of the solution *in vacuo* left an amorphous sodium salt, which showed an activity of 20 u./mg. when assayed against *Staph. aureus* and 2·2 u./mg. when assayed against *Salm. typhi*. The ultraviolet absorption spectrum of the sodium salt showed an inflection in the region of 260 mμ. ($E^{1\%}_{1\,cm.}$ = 124).

### *Hydrogenation of cephalosporin C*

Hydrogenation of cephalosporin C sodium salt (4·7 mg.) in 2·6 ml. of water was carried out in the presence of 12 mg. of 10% Pd on charcoal (L. Light and Co. Ltd.) in a Warburg respirometer. There was a rapid absorption of $H_2$ during the first 30 min., after which the reaction became much slower. After 2 hr. when about 0·9 mole (200 μl.) of $H_2$ had been absorbed the experiment was stopped. The mixture was filtered and the filtrate evaporated to dryness *in vacuo*, leaving a non-crystalline residue (2·2 mg.). The ultraviolet absorption spectrum of the crude product was similar in shape to that of cephalosporin C, but the extinction at 260 mμ. ($E^{1\%}_{1\,cm.}$ = 144) was somewhat lower. When subjected to chromatography on paper in butanol–acetic acid, or to ionophoresis on paper at pH 7, the product behaved like cephalosporin C. After hydrolysis with N-HCl at 105° for 16 hr. it gave two major spots on a two-dimensional paper chromatogram, run with butanol–acetic acid and phenol respectively, in positions corresponding to α-aminoadipic acid and glycine. When the hydrolysate was treated with $Br_2$ water a small amount of a new substance was formed which behaved like penicillaminic acid on a paper chromatogram run with butanol–acetic acid and also on ionophoresis on paper at pH 7.

### *Hydrogenolysis of cephalosporin C*

*Experiment* 1. A solution of cephalosporin C sodium salt (50 mg.) in water (5 ml.) was placed in a flask fitted with a reflux condenser. Raney nickel (about 300 mg.), prepared by the method Pavlic & Adkins (1946), was added and the flask immersed in an oil bath at 160°. After 15 min. the mixture was cooled and the Raney nickel removed by filtration and washed 3 times on the filter with 0·5 ml. of water. The filtrates were combined, and shaken twice with 1 vol. of 1% (w/v) oxine in $CHCl_3$ and then twice with 1 vol. of $CHCl_3$ alone. The aqueous solution was centrifuged clear and evaporated to dryness *in vacuo*, leaving 35 mg. of a gum. This product showed no absorption maximum between 220 and 300 mμ. The value of $E^{1\%}_{1\,cm.}$ at 260 mμ. was 20·0. The corresponding value for the starting material was 180. When a sample of the product (100 μg.) was subjected to ionophoresis on paper at pH 7 (14 v/cm. for 2·5 hr.) and the paper sprayed with ninhydrin, a strong spot was obtained due to acidic material with a mobility similar to that of cephalosporin C, and a weaker neutral spot. The main part of the product was separated into a neutral fraction (4 mg.) and an acidic fraction (17 mg.) in the four-compartment cell. Chromatography on paper in butanol–acetic acid showed that both fractions were complex. The neutral fraction showed a major spot with an $R_F$ value slightly less than that of glycine and other spots in the glycine, alanine and valine positions. The acidic fraction showed a major spot with an $R_F$ value (0·42) between those of α-aminoadipic acid and valine. After hydrolysis with N-HCl at 105° for 16 hr. both fractions gave two major spots, the first in the position of α-aminoadipic acid (and alanine) and the second in the position of valine. There were also two weaker spots, one in the position of glycine and the other with an $R_F$ value of about 0·13.

*Experiment* 2. Cephalosporin C sodium salt (500 mg.) was subjected to hydrogenolysis with Raney nickel (about 1 g.) under the conditions described in Expt. 1. When a sample of the product was hydrolysed in N-HCl at 105° the amount of valine formed (as estimated by paper chromatography in butanol–acetic acid) was less than that obtained in the preceding experiment. The major part of the material was therefore treated further with Raney nickel (4 g.) under similar conditions. The final product was hydrolysed in refluxing 2 N-HCl (50 ml.) for 12 hr. The solution was shaken twice with 1 vol. of ether, and the ether evaporated to yield a gum which has not been identified. The aqueous phase was evaporated to dryness in a rotary evaporator (Craig *et al.* 1950) and the residue (300 mg.) was subjected to ionophoresis in the four-compartment

cell until the current fell to 30 mA with a potential of 500 V. The contents of the acetic acid compartment were evaporated in a rotary evaporator and yielded 150 mg. of acidic material. The contents of the specimen compartment yielded 50 mg. of neutral material. The acidic material showed a single ninhydrin-positive spot in the position of α-aminoadipic acid on paper chromatograms developed with butanol–acetic acid or with phenol. The neutral material showed spots in the positions of glycine, alanine and valine respectively in the same solvent systems. The glycine spot had the typical red tint given by authentic glycine with the ninhydrin reagent used (Woiwod, 1949).

The acidic material was chromatographed on a column (0·9 cm. × 36 cm.) of Dowex 1 × 10 (200–400 mesh) in the acetate form, elution being carried out with 0·5 N acetic acid (Fig. 5). The flow rate was 4 ml./hr. and 1·0 ml. fractions were collected. A white crystalline precipitate separated from fractions 18–20 inclusive and was removed by filtration and dried (37 mg.). It had m.p. 207–208°, unchanged by admixture with an authentic sample of D-α-aminoadipic acid (m.p. 207–208°) kindly supplied by Dr J. P. Greenstein; $[\alpha]_D^{20} - 25°$ in 6 N-HCl (*c*, 3·0). (Found: C, 44·6; H, 6·8; N, 8·5. $C_6H_{11}O_4N$ requires C, 44·8; H, 6·8; N, 8·7%.)

The neutral material was chromatographed on a column (0·9 cm. × 41 cm.) of Dowex 50-X4 in the acid form, elution being carried out with N-HCl (Fig. 4). The flow rate was 6 ml./hr. and 1·5 ml. fractions were collected. Photometric ninhydrin determinations (Moore & Stein, 1954) were made on samples (0·1 ml.) from selected fractions. Fractions 40–45 inclusive were evaporated to dryness *in vacuo* and yielded a crystalline residue. The residue was dissolved in 0·5 N acetic acid (1·5 ml.) and passed through a short column (0·9 cm. × 4 cm.) of Dowex 1 × 10 (200–400 mesh) in the acetate form. When the column was washed with 0·5 N acetic acid all the amino acid emerged in the first 3 ml. of effluent. Evaporation *in vacuo* left a crystalline residue which was recrystallized from aqueous ethanol. The infrared spectrum of the product was identical with that of a sample of authentic glycine between 3 and 8·9 μ. The bands between 9 and 14·5 μ. were indistinguishable in number and intensity from those of the authentic sample, but in most cases showed a displacement of about 0·05 μ. towards a shorter wavelength. This phenomenon may be due to the fact that glycine can crystallize in two forms (Bernal, 1931).

Fractions 53–58 (estimated by the photometric ninhydrin procedure to contain the equivalent of 5·4 mg. of alanine) were pooled and evaporated *in vacuo* to yield a crystalline residue (7 mg.) of hydrochloride, $[\alpha]_D^{20} + 8°$ in 6 N-HCl (*c*, 1·0). Fischer & Raske (1907) reported $[\alpha]_D^{20} + 10·4°$ for L-alanine hydrochloride. The solution from the polarimeter tube was evaporated to dryness and the residue dissolved in 0·5 N acetic acid and passed through a short column of Dowex 1 acetate to remove hydrochloric acid. The resulting free amino acid was crystallized from aqueous ethanol. The infrared spectrum of the product was identical with that of an authentic sample of L-alanine.

Fractions 60–64 (estimated by the photometric ninhydrin procedure to contain the equivalent of 11·5 mg. of valine) were pooled and evaporated *in vacuo* to yield 15 mg. of a crystalline hydrochloride, $[\alpha]_D^{20} - 5°$ approx. in 6 N-HCl (*c*, 0·4). Fischer (1906) reported $[\alpha]_D^{20} + 28·8°$ for L-valine in 5·5 N-HCl. The hydrochloride was converted into the free amino acid by the method used for alanine. After crystallization from aqueous ethanol the product showed an infrared spectrum which was identical with that of authentic DL-valine. (Found: C, 50·9; H, 9·0. $C_5H_{11}O_2N$ requires C, 51·3; H, 9·4%.)

## SUMMARY

1. Cephalosporin C is relatively stable in dilute acid at room temperature, but is rapidly inactivated at pH 12. Inactivation in alkaline solution at 20° is accompanied by the loss of an absorption band at 260 mμ. and the liberation of two acidic groups. Hydrolysis with alkali at 100° results in the formation of substances which behave like α-aminoadipic acid and glycine respectively on paper chromatograms.

2. Cephalosporin C is inactivated by an enzyme in partly purified preparations of penicillinase from *B. cereus*, with loss of its absorption band at 260 mμ. and liberation of one acid group.

3. Hydrolysis of cephalosporin C with dilute acid at 105° results in the liberation of carbon dioxide, volatile base, D-α-aminoadipic acid and traces of glycine. Little or no penicillamine appears to be formed. Hydrolysis of the dinitrophenyl derivative of cephalosporin C results in the liberation of dinitrophenyl-α-aminoadipic acid.

4. Cephalosporin C absorbs 1 mole of hydrogen in the presence of a palladium–charcoal catalyst, yielding a product which has little antibacterial activity but which still shows an absorption maximum at 260 mμ. Hydrolysis of the product with dilute acid at 105° results in the formation of α-aminoadipic acid and glycine. Treatment of the hydrolysate with bromine water yields a small amount of a substance which behaves like penicillaminic acid on paper chromatograms.

5. Hydrolysis with dilute acid at 105° of the material obtained by hydrogenolysis of cephalosporin C with Raney nickel yields D-α-aminoadipic acid, valine which is extensively racemized but which appears to contain an excess of the D-form, and smaller amounts of L-alanine and glycine. In yielding valine, alanine, and glycine after hydrogenolysis cephalosporin C resembles the penicillins.

6. The dinitrophenyl and benzoyl derivatives of cephalosporin C are more active against *Staph. aureus* and less active against *Salm. typhi* than the parent compound.

We are grateful to Miss M. Read and Mr O. Boys for expert technical assistance.

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# The Separation of Substances and Estimation of their Relative Molecular Sizes by the use of Columns of Starch in Water

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(*Received* 29 *August* 1955)

Starch is frequently used as an inert support in partition chromatography. Departure from ideal behaviour has been interpreted on the basis of adsorption (Moore & Stein, 1948) or the effect of hydrated starch on solubility in the stationary phase (Martin, 1950). It has been assumed that starch grains are uniformly permeable to all solutes. The present study demonstrates that solutes vary greatly in their ability to penetrate hydrated starch grains, and that the extent of penetration is determined primarily by molecular size. Columns of starch in water form a new type of partition system, based on this molecular-sieve effect, and from these columns certain types of substances are eluted in order of decreasing molecular weight. They can be used for separation of mixtures, and under some circumstances the molecular dimensions of solutes may be determined. Some of these findings have been given in a preliminary communication (Lathe & Ruthven, 1955).

## METHODS

*Starches.* Trials were made with potato, wheat, maize and rice starches, as supplied by British Drug Houses Ltd. Because of the ease of washing and the speed of flow, potato starch was used for most columns of unaltered starch.

*Swollen maize starch.* Because of the relative uniformity of its grains and since they do not burst on heating, maize starch was selected for the preparation of columns of swollen starch. The unwashed, undried maize starch (250 g.) was suspended in 2·5 l. of distilled water, with mechanical stirring which was continued throughout the preparation. The volume of starch was estimated by centrifuging 10 ml. of the mixture for 3 min. at 1250 *g*. The solution was warmed (by a thermomantle) from room temp. to 61° during 75 min., without an appreciable change in the starch volume. During the following 75 min. the temp. was raised to 68° and the procedure was stopped when the starch volume had increased to three times the original. The mixture was poured into 7 l. of distilled water, allowed to stand for 24 hr., and the slightly turbid supernatant was removed. The mixture was kept at 4°, and, in the presence of preservative, did not appear to deteriorate over several months.

A few experiments have been conducted with starch swollen by standing overnight at 4° in the presence of KSCN. 12·9% KSCN resulted in an increase of 0·15 vol., 14·1% KSCN 1·3 vol., and 14·8% KSCN 4·5 vol. The second of these preparations behaved in several experiments much like starch swollen threefold by heat. Although swelling the starch with salt is easy to carry out and to control, the repeated washing to remove the salt is laborious.

*Preservative.* Early columns had to be discarded in a few days because of gas formation, presumably owing to bacteria. This was avoided in later columns by addition of $CHCl_3$ (about 0·6%) to the water or buffer. Saturation with thymol was also satisfactory for preservation, but it interfered with the anthrone reaction.

*Preparation of columns.* Untreated potato-starch columns of 20 or 75 g. were set up as follows. The commercial, undried starch (20 g.) was suspended by hand in 100 ml. of water or buffer. After standing for 10 min. the supernatant was drawn off. This was repeated twice more, and the final volume made to about 100 ml. The slurry was delivered in approximately 10 ml. amounts into a 16 mm. (internal diameter) chromatography tube the lower end of which was narrowed and filled with a small cotton plug. Each sample was allowed to stand until it had almost completely settled (5–10 min.) and the supernatant was

sucked off. After all the starch had been added, the column was allowed to stand overnight. Approximately 10 ml. of fluid was then run through the column, which now had a height of 15–16 cm. The material (0·5–5 mg.) to be studied was dissolved in 0·5 ml. of water or buffer and placed on top of the column and was allowed to run into the starch. It was eluted with more water or buffer at the rate of 4 ml./hr.

Columns of swollen starch were prepared from the swollen maize-starch mixture. The starch suspension was remixed to uniformity, and a 65 ml. portion (or sufficient to yield 25–30 ml. of packed starch) was centrifuged for 2 min. at 1500 *g*. The supernatant was poured off. The starch was washed three times with 35 ml. portions of buffer by suspension and centrifuging. Finally a homogeneous slurry was prepared by adding gradually, with stirring, about 30 ml. of buffer. This was pipetted into a glass column of 16 mm. diameter and 70 cm. length; the tip of the pipette was raised during this process so that it was a few mm. above the liquid surface. The sides of the column were washed down with more buffer, which was then allowed to flow through the column overnight or until the height of the column remained constant (about 16 cm.). After addition of the solute in 0·5 ml. of buffer the columns were eluted at about 3 ml./hr.

It was not possible to set up swollen starch columns with constant weights of starch. As a result of this, as well as other factors, there was some variation in the size and behaviour of different columns and a means of comparing different columns was necessary. It was found satifactory to 'calibrate' swollen starch columns on the basis of the elution volume (defined below) of haemoglobin, which was 15 ml. for a 35 ml. column. The elution volumes of other substances reported in this paper have been adjusted proportionately on this basis, except where haemoglobin was not run. In these cases the elution volume has been adjusted on the basis of the volume of the column. After adjustment the error between replicate determinations was less than 0·5 ml. except for smaller molecules. For instance, the elution volumes of cyanocobalamin on six columns of threefold swollen starch, after adjustment to a haemoglobin elution volume of 15 ml., were 28·0, 29·2, 26·7, 26·4, 26·5, 26·7 and 27·3 ml. The error is of a higher order here, but the columns are unsuitable for separating molecules of this size.

Cellulose columns were prepared from 10 g. of Whatman Cellulose Powder, Standard Grade (containing 4·4% w/v water) by a method similar to the one used for unheated starch.

The elution volume is the total volume of liquid which flows from the column between the addition of the solute and the emergence of the maximum concentration of solute.

*Determination of 'column water'.* The water content of commercial potato starch (about 20% w/v) was determined accurately for each lot by drying a sample to constant weight in a vacuum desiccator over $H_2SO_4$. The total amount of water held by a column was determined by weighing a loaded column and by subtracting the calculated dry weight of the starch and the weight of the empty column.

*Buffers.* Phosphate buffer (0·05 M) was 0·89% (w/v) $Na_2HPO_4,2H_2O$. Borate and acetate buffers were prepared according to Cole (1933).

*Determination of substances.* Glucose, streptomycin, sucrose, inulin and glucosamine were estimated by a copper-reduction method (King, 1951). Sucrose and inulin were first hydrolysed with 0·1 N-HCl for 10 min. at 100°. Raffinose was estimated by determination of the galactose (King, 1951) present after yeast fermentation of an acid hydrolysate (0·1 N-HCl at 100° for 10 min.). Other sugars and polysaccharides were determined by the anthrone method as modified by Loewus (1952), scaled down to 2·25 ml., and carried out in Wasserman tubes. The urea–resorcinol method of Preedy (1954), with the omission of protein precipitation, was also used for inulin when the anthrone method gave excessive blank values.

Haemoglobin was estimated by the extinction at 578 mμ., cyanocobalamin at 362 mμ., bacitracin A at 254 mμ., cytochrome *c* at 520 mμ. and myoglobin at 580 mμ. The extinction at 280 mμ. served for colourless proteins, Borsook's peptide A, benzoic acid, aniline, chloramphenicol and thiamine. Amino acids were determined qualitatively by spotting on filter papers which were stained by dipping in a 0·25% ninhydrin solution in acetone, and were allowed to dry at room temp. Paper chromatography with *n*-butanol–acetic acid–water (40:10:50, by vol.), according to Partridge (1948), distinguished the amino acids, and a solvent mixture of *n*-propanol–ethyl acetate–water (6:1:3, by vol.) was used to characterize sugars (Buchan & Savage, 1952). Urea was determined by nesslerization (King, 1951), and carbonic anhydrase according to Richter & Hullin (1951).

*Materials.* Crystalline cyanocobalamin was a gift from Dr E. Lester Smith, of Glaxo Laboratories Ltd. Maltotriose, -tetraose, -pentaose, α- and β-Schardinger dextrins (*cyclo*malto-hexaose and -heptaose), soluble laminarin, amylose and amylopectin were provided by Dr W. J. Whelan. Bacitracin A was provided by Dr E. P. Abraham, and polymyxin B sulphate by Dr S. Wilkinson of The Wellcome Research Laboratories. Crude goat β-lactoglobulin was given by Dr B. A. Askonas, and was purified by precipitation at a concentration of $(NH_4)_2SO_4$ between 62 and 72%. Crystalline insulin (Boots), protamine (Light), plasma albumin (Armour), pig pepsin (Armour), ox trypsin (Armour), inulin (Gurr), and most amino acids and peptides were obtained commercially. Haemoglobin was prepared by lysing washed adult red blood cells in distilled water. Cytochrome *c* was prepared from horse heart by the method of Keilin & Hartree (1945). Peptide A of Borsook, Deasy, Haagen-Smit, Keighley & Lowy (1949) was prepared from Witte's peptone. Plasma globulin was prepared from human serum by precipitation with 50 vol. of 42% (w/v) $Na_2SO_3,7H_2O$. Human red blood cells, lysed in 1–2 vol. of water, provided carbonic anhydrase. Myoglobin was extracted from ox-skeletal muscle by an adaptation of the method of Keilin & Schmid (1948).

*Molecular weights.* These have been calculated for substances of known composition. The values for all other substances have not been determined, but are taken from the current literature.

## RESULTS

### *Composition of potato-starch column*

There were minor variations in the water content of potato starch, and in the dimensions of columns. The following is a typical example. A column of

20 g. of potato starch initially containing 19% (w/w) water was set up in water, and after contraction it occupied 25·8 ml., and weighed 32·0 g. Thus for every 10 g. of undried starch in the column there was 7·9 ml. of water. Colloidal carbon (indian ink) was eluted at 4·1 ml. (range 4–4·25 ml.)/10 g. of starch. Under the microscope it was seen that carbon particles did not penetrate starch granules. Thus the elution volume of carbon measured the mobile phase outside the grains. Accordingly, the mobile phase was 4·1 ml., the hydrated grains occupied 8·8 ml. and contained 3·8 ml. of water.

### *Behaviour of uncharged molecules on untreated starch*

A number of uncharged substances, chiefly sugars, were eluted from 75 g. potato-starch columns, in the positions shown in Fig. 1. These are shown in a standardized way, all 'peaks' being recorded at the same height, in order to avoid differences due to the variety of methods of determination, and the varying amounts of material used. Urea, which is not shown in the figure, was eluted at 73·5 ml. Of three monosaccharides, galactose and glucose were eluted together at 49 ml., and fructose was slightly retarded (51 ml.). One curve is given for three disaccharides, since sucrose, maltose and lactose were not separated. Higher sugars of the maltose series showed some unusual behaviour with regard to their positions in a few experiments and, with the exception of maltotriose, there was marked skewing of the curves. They require further study, but are included for completeness. Maltotriose was eluted after raffinose (41 and 37 ml. respectively). Maltotetraose (pH 8·6 in phosphate) spread widely over the region of tri- and di-saccharides (37–49 ml.). Maltopentaose (pH 8·6 in phosphate) had a peak at 37 ml., but trailed badly through the disaccharides region.

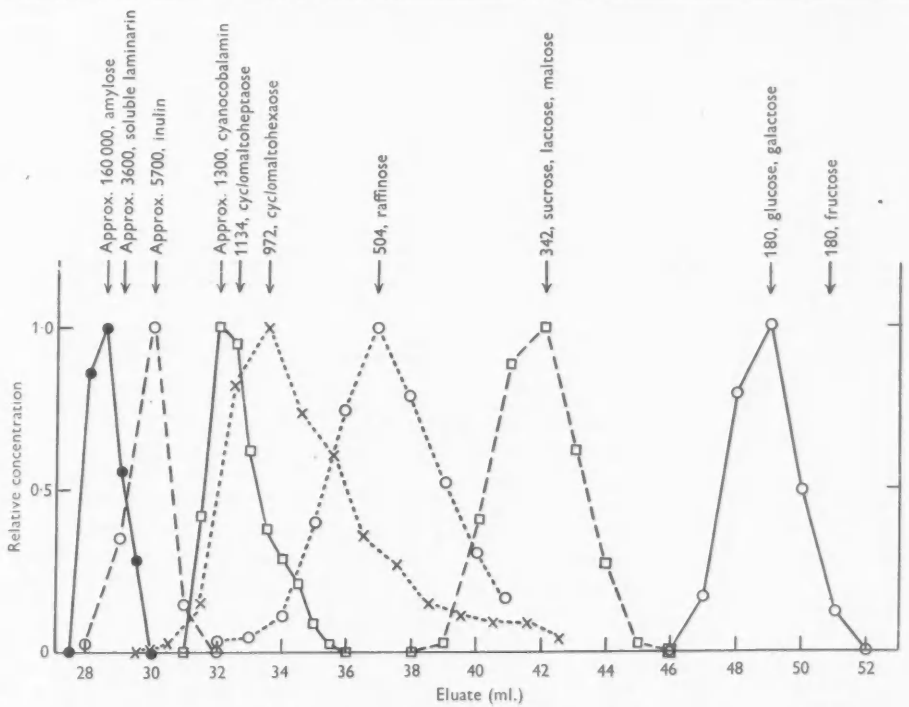


Fig. 1. Elution of neutral molecules in order of descending molecular weights, from columns of 75 g. of untreated potato starch in water. The points of maximum concentration have been adjusted to an arbitrary height. ●——●, Amylose; ○— — —○, inulin; □——□, cyanocobalamin; ×- - - - -×, *cyclo*maltohexaose; ○- - - -○, raffinose; □— — —□, sucrose; ○——○, glucose. The positions of peaks of soluble laminarin, *cyclo*maltoheptaose and fructose are also shown.

Table 1. *Volume of elution of small charged molecules from columns of 20 g. of potato starch in various solutions*

Four uncharged molecules are included as markers. Elution volume is defined in the Methods section.

| | Mol. wt. | Elution volume (ml.) pH 8·5, 0·05 M phosphate | pH 8·5, 0·05 M phosphate, 0·25 M-NaCl | pH 10·0, 0·05 M borate | pH 2·2, 0·01 N-HCl | pH 2·2, 0·01 N-HCl, 0·25 M-NaCl |
|---|---|---|---|---|---|---|
| Inulin | 5700 | 9·0 | 9·0 | — | 8·0 | 8·75 |
| Sucrose | 342 | 11·5 | 12·0 | — | 12·5 | 12·0 |
| Glucose | 180 | 14·0 | 14·0 | — | 13·0 | 14·0 |
| Urea | 60 | 19·0 | 19·0 | — | — | — |
| Galacturonic acid | 196 | 13·0 | 13·3 | — | 15·5 | 14·3 |
| Glucosamine | 179 | 17·0 | 15·5 | — | >22·0 | 14·8 |
| Benzoic acid | 122 | 18·0 | 19·3 | — | >31·0 | >31·0 |
| Aniline | 93 | 28·0 | 29·0 | — | >30·0 | 17·5 |
| Chloramphenicol | 323 | 25·0 | 24·0 | — | 24·0 | 24·0 |
| Streptomycin | 582 | >13·0 | 17·5 | — | >16·0 | 12·0 |
| Thiamine | 337 | 20·0 | 17·5 | — | — | — |
| Riboflavin | 376 | >25·0 | >25·0 | — | — | — |
| Oxidized glutathione | 612 | 10·5* | — | — | — | — |
| Reduced glutathione | 307 | 11·0† | — | — | 17·0‡ | — |
| Glycylleucine | 188 | 11·5 | — | — | — | — |
| Diglycylglycine | 189 | 12·0 | — | — | — | — |
| Leucylglycine | 188 | 12·5 | — | — | — | — |
| Glutamic acid | 163 | 12·0 | 13·0 | — | 25·5 | 15·5 |
| Aspartic acid | 133 | 13·0 | 13·5 | — | 24·0 | 15·5 |
| Leucine | 131 | 13·5 | 13·5 | — | — | — |
| Proline | 115 | 13·5 | — | — | — | — |
| Alanine | 89 | 14·0 | 14·5 | — | 25·0 | 14·5 |
| Valine | 117 | 14·7 | — | — | — | — |
| Norleucine | 131 | 15·2 | — | — | — | — |
| Glycine | 75 | 15·5 | 15·5 | — | 28·0 | 16·5 |
| Threonine | 119 | 15·5 | — | — | — | — |
| Serine | 105 | 15·5 | — | — | — | — |
| Cystine | 240 | 16·0 | — | — | — | — |
| Methionine | 149 | 16·5 | — | — | — | — |
| Lysine | 146 | 17·5 | 15·5 | 13·5 | 29·0 | — |
| Folic acid | 441 | 17·5 | — | — | — | — |
| Histidylhistidine | 293 | 18·0 | 19·0 | — | — | — |
| Histidine | 155 | 18·5 | 17·0 | 15·0 | — | — |
| Tyrosine | 181 | 20·5 | 21·5 | — | — | — |
| Arginine | 174 | 23·5 | 19·5 | 20·0 | >31·0 | 18·5 |
| Tryptophan | 204 | 29·0 | 33·5 | — | — | — |

* 0·05 M Borate, pH 8·5.
† 0·05 M Borate, pH 8·5, 0·06 M thioglycollate.
‡ Saturated benzoic acid, pH 3·0.

Table 2. *Behaviour of large charged molecules on columns of 20 g. of potato starch in phosphate buffer*, pH 8·5

Sodium chloride when present was 0·25 M.

| Protein | Mol.wt. | Elution volume (ml.) Salt present | Salt absent |
|---|---|---|---|
| Haemoglobin | 67 000 | 8·5 | 8·5 |
| Cytochrome *c* | 13 000 | 8·5 | 8·8 |
| Insulin* | 12 000? | 8·5 | >19 |
| Peptide A† | ? | 9·0 | 9·0 |
| Protamine | 3 000 | 10·0 | >29 |
| Polymyxin B | Approx. 1 200 | 13 | >29 |

* In 0·01 N-HCl.
† Borsook *et al.* (1949).

*Behaviour of charged molecules on untreated starch*

Preliminary trials of acids and bases of low molecular weight indicated that factors other than molecular weight affected their movement on starch columns. The elution volume of charged molecules, unlike sugars, was altered by change of hydrogen-ion concentration. The effect of variations of pH, with and without additional sodium chloride, were examined for two acid–base pairs (glucosamine–galacturonic acid; aniline–benzoic acid), some naturally occurring acid and basic substances, and a series of amino acids and di- and tri-peptides, on 20 g. starch columns (Table 1). Retardation of charged molecules appeared to be

minimal at pH 8·5 in the presence of salt. The effect of negative charge was examined in 0·05 M phosphate buffer at pH 7·0. Glucuronic acid was eluted at 12 ml., galacturonic acid at 12·5 ml., glucose at 14 ml., and glucuronic acid lactone at 15 ml. A few large charged substances were run on columns of 20 g. of untreated potato starch at pH 8·5 in phosphate buffer with 0·25 M-NaCl (Table 2). When compared with the 75 g. columns of Fig. 1 the elution volumes of the neutral markers, inulin, sucrose, glucose and urea from 20 g. columns were found to be approximately 1 ml. greater than anticipated. This difference is probably due to closer packing of the starch on the large columns.

*Swollen maize starch*

To increase the extent to which larger molecules penetrated the starch grains they were swollen by heating in water. Maize starch, granules of which do not burst on heating, were treated until the volume was three times the original. A typical 'standard' column of threefold swollen maize starch, prepared as described in Methods, occupied 35 ml. The mobile phase (measured with colloidal carbon) occupied 11·5 ml., and the volume of stationary hydrated-starch phase was 23·5 ml. The stationary phase contained 5·7 g. of dry starch, and had the following composition: 24·3 % (w/v) starch and 93 % (w/v) water.

*Behaviour of large molecules on swollen starch columns*

The positions in which a number of large neutral and charged molecules were eluted from standard columns of this type at pH 8·5 in 0·025 M borate buffer containing 0·025 M-KCl are shown in Fig. 2. Cytochrome *c*, protamine and polymyxin B could not be eluted from swollen maize-starch columns in this solvent. In view of the basic character of these substances the effect of salt concentration on the

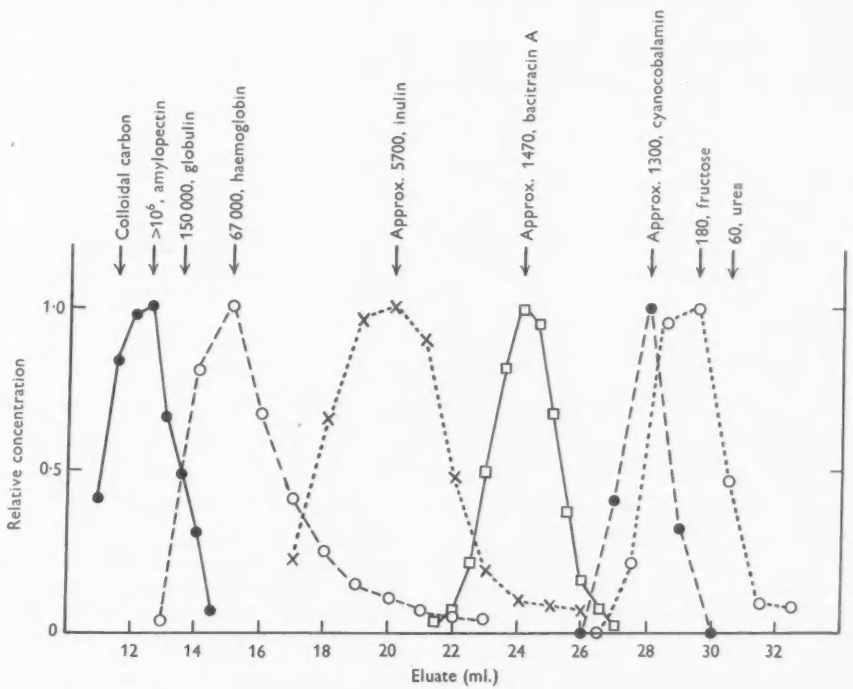


Fig. 2. Elution of large molecules in order of descending molecular weights, from standard columns of heat-swollen maize starch in 25 mM borate and 25 mM-KCl. The points of maximum concentration have been adjusted to an arbitrary height. ●——●, Amylopectin; ○— — —○, haemoglobin; ×- - - - -×, inulin; □——□, bacitracin A; ●— — —●, cyanocobalamin; ○- - - -○, fructose. The positions of peaks of globulin and urea are also shown.

rate of movement of cytochrome was examined (Table 3). As a result, whenever possible proteins were examined in 0·05M phosphate buffer at pH 8·5, containing 0·25M-NaCl. The results from a number of proteins of mol.wt. 150 000 to 12 000 are given in Table 4. Protamine could not be eluted from swollen starch in this solution, and polymyxin B was retarded. For reasons of solubility and stability insulin and trypsin were examined at pH 2·2 in presence of 0·25M-NaCl.

*Recoveries*

Low recoveries of some sugars were observed on a number of columns of unswollen starch, namely glucose 70 and 86%, raffinose 57%, maltohexaose 60%. Sucrose (114 and 106%), maltotriose (100%), inulin (97%), glucosamine (98%) and galacturonic acid (95–99%) were more satisfactory. Proteins were recovered 80–100% from both unswollen potato starch and swollen maize starch.

Table 3. *Elution volume of cytochrome c on standard columns of swollen starch in different solutions*

| pH | Solution | Elution volume (ml.) |
|---|---|---|
| 8·5 | 25 mM borate, 25 mM-KCl | Approx. 45 |
| 8·5 | 50 mM borate | Held |
| 8·5 | 50 mM borate, 50 mM-KCl | 25 |
| 8·5 | 50 mM borate, 50 mM-KCl, 0·25M-NaCl | 18 |
| 8·5 | 12·5 mM phosphate | Held |
| 8·5 | 25 mM phosphate | 26 |
| 8·5 | 50 mM phosphate | 18 |
| 8·5 | 50 mM phosphate, 0·25M-NaCl | 18 |
| 6 | Water | Held |
| 6 | 25 mM-NaCl | 18 |

Table 4. *Volume of elution of proteins from standard columns of threefold swollen maize starch*

Concentrations of buffers and salts were: phosphate, 50 mM; borate, 25 mM; acetate, 50 mM; HCl, 0·01N; KCl, 25 mM; NaCl, 0·25M.

| Protein | Solution | Mol.wt. | Elution volume (ml.) |
|---|---|---|---|
| Globulin | pH 8·5, borate; KCl | 150 000 | 14·2 |
| Albumin | pH 8·5, phosphate; NaCl | 65 000 | 14·5 |
| Haemoglobin | pH 8·5, borate; KCl | 67 000 | 15·0 |
| | pH 8·5, phosphate; NaCl | — | 15·0 |
| Carbonic anhydrase | pH 8·5, phosphate; NaCl | 30 000? | 16·5 |
| Lactoglobulin | pH 8·5, phosphate; NaCl | 38 000 | 16·7 |
| Myoglobin | pH 8·5, phosphate; NaCl | 17 000? | 17·1 |
| Pepsin | pH 5·0, acetate; NaCl | 35 000 | 17·3 |
| Trypsin | pH 3·45, acetate; NaCl | 15 000 | 18·5 |
| | pH 2·2, HCl; NaCl | — | 18·1 |
| Cytochrome *c* | pH 3·45, acetate; NaCl | 13 000 | 18·5 |
| | pH 8·5, phosphate; NaCl | — | 18·8 |
| Insulin | pH 2·2, HCl; NaCl | 12 000? | 20·0 |
| Inulin | pH 8·5, borate; KCl | Approx. 5 700 | 20·0 |
| Polymyxin B | pH 3·5, acetate; NaCl | Approx. 1 200 | 30·0 |

*Cellulose columns*

Cellulose columns occupied 33 ml. per 10 g. of undried cellulose and contained 29·4 ml. of water. Haemoglobin was eluted at 26·0 ml., glucose and urea at 28·0 ml. Assuming that haemoglobin did not penetrate the cellulose fibres it may be calculated from its elution volume and from the $R$ value of urea, relative to haemoglobin, that urea was distributed in a stationary phase of 1·96 ml. [$R=(M/S)/(1+M/S)$, where $M$ and $S$ are the volumes of mobile and stationary phases respectively].

*Role of solubility partition*

The possibility that these separations were due to a partition between water and hydrated starch, based on solubility factors (Martin, 1950), has been examined. The stationary phase of unswollen hydrated potato starch has the composition 92% (w/v) starch and 43% (w/v) water. As an approximation to this composition, 87% (w/v) sucrose was prepared, and the ratio of solubilities in water and in this solution were determined for some slow- and fast-moving solutes. In order to calculate the $R$ values which would result from the partitions

indicated by the ratio of solubilities in water and 87% (w/v) sucrose solution, account must be taken of the relative volumes of the mobile and stationary phases. The volume of mobile phase is 4·1 ml./10 g. of starch, while that of stationary phase is intermediate between 3·8 ml. (the volume of water in the phase) and 8·8 ml. (the total volume occupied by the hydrated grains). $R$ values have been calculated for assumed stationary phase volumes of 6·4 and 8·8 ml. and are compared with the observed $R$ values in Table 5.

Smaller molecules, and in particular those of mol.wt. less than about 1000, are retarded to some extent on starch columns. It may also be demonstrated directly that to a varying degree they are removed from solutions in contact with hydrated starch. There are two possible explanations. First, all solutes may be regarded as remaining outside the starch granules, and retardation on the column (or removal from solution) may be attributed to adsorption to the grain surface. Secondly, the retardation on the columns may be due to a penetration of the starch granules, the smaller substances being distributed inside as well as outside the granules. The simultaneous occurrence of adsorption and penetration is, of course, not excluded.

Table 5. *Comparison of observed and theoretical* R *values*

Theoretical $R$ values were calculated from the formula $R=[(M/S)\times\text{solubility quotient}]/\{1+[(M/S)\times\text{solubility quotient}]\}$, where $M$ is the known volume of mobile phase, and $S$ is an arbitrarily assumed volume of stationary phase, in ml., for a column containing 10 g. of undried starch.

| | Solubility in water (w/v) | Solubility in 87% sucrose (w/v) | Solubility quotient (water/sucrose) | Calc. $R$ value | | Obs. $R$ value |
|---|---|---|---|---|---|---|
| | | | | $S=6·4$ | $S=8·8$ | |
| Cyanocobalamin | 1·5 | 0·7 | 2·1 | 0·57 | 0·50 | 0·91 |
| Raffinose | 17 | 2 | 8·5 | 0·85 | 0·80 | 0·75 |
| Sucrose | 91 | 4 | 22·8 | 0·94 | 0·91 | 0·62 |
| Glucose | 67 | 10 | 6·7 | 0·81 | 0·76 | 0·57 |
| Glycine | 23 | 11 | 2·1 | 0·57 | 0·50 | 0·52 |
| Urea | 51 | 36 | 1·4 | 0·47 | 0·40 | 0·42 |

As an alternative method of investigating the role of solubility partition, we examined the effect on the $R$ values of making the mobile phase more nearly of the same composition as the stationary phase. This was achieved by substituting concentrated sugar solutions for water in the preparation of the columns. There was no change in the $R$ values of haemoglobin, cyanocobalamin and inulin on starch columns when 48% (w/v) glucose was used as mobile phase, and no change in $R$ of cyanocobalamin, glycine and urea on columns in 87% (w/v) sucrose.

## DISCUSSION

Starch grains are impermeable to particles of colloidal carbon, which were eluted from a column of 10 g. of potato starch in 4·1 ml. Since the column contained 7·9 ml. of water, it is concluded that during elution water flows about the starch grains, rather than through them. The fact that large molecules [amylose, laminarin, inulin (Fig. 1); haemoglobin, cytochrome (Table 2)] were eluted in a volume that was almost the same as that for carbon indicates that none of these large molecules penetrates untreated starch granules to an appreciable extent. This may also be demonstrated directly by adding a measured amount of solute of high mol.wt. (e.g. inulin) to known amounts of starch and water. The resulting concentration of solute in the water shows that for every 10 g. of starch there is approximately 4 ml. of water in which inulin does not distribute itself.

Moore & Stein (1952) have stressed the adsorptive properties of cellulose and starch, and they support this view in part by the separation of alanine and glycine on columns of starch in water, without the aid of an immiscible solvent (Moore & Stein, 1948). Adsorption by ionic forces can be excluded as an explanation of the separation of uncharged molecules (Fig. 1), the movement of which is unaffected by changes of pH and salt concentration (Table 1). Hydrogen bonding might play a part. However, hydrogen bonding would be more important for large molecules than small ones, whereas it is the latter that are retarded on columns of starch in water. Thus it is improbable that the retardation of small uncharged molecules can be explained on the basis of adsorption to the granule surface by ionic or secondary linkages. For these molecules penetration of the granules seems more probable. This would have the effect of separating molecules which penetrate the granules from those which do not, the latter being eluted first.

In discussing the factors which may determine the distribution of solutes in partition systems, Martin (1950) suggested that under some circumstances cellulose (or starch) should not be regarded merely as a support for the stationary phase. 'The

stationary phase in a cellulose chromatogram should be compared with, say, a strong solution of glucose, or better some soluble polysaccharide, rather than with water saturated with the organic phase.' According to this argument, solutes could partition themselves on the basis of solubility factors, between water and the hydrated starch grains of starch and water columns. There are two types of evidence in the present study which are inconsistent with this theory.

The first concerns the relation between solubilities and *R* values. In three cases (Table 5) there is poor agreement between the observed *R* values and the *R* values calculated from the relative solubilities in water and in 87% (w/v) sucrose solution. In particular, cyanocobalamin and glycine, which should behave similarly according to solubility-partition theory, have observed *R* values of 0·91 and 0·52 respectively. Secondly, on the basis of solubility theory, all solutes should move with the same *R* value, about 0·4, if the ambient and stationary phases had the same composition. Attempts to change the observed *R* values in this direction, by increasing the carbohydrate content of the moving phase (to 87%, w/v, sucrose), were without effect. Thus solubility-partition theory does not explain the main aspects of starch and water columns, and especially the great differences in behaviour of large and small molecules. Solubility partition may play a part, however, in determining the relative movement of smaller molecules (Table 2). This theory could only be applied to molecules which can move unhindered between mobile and stationary phases.

Cyanocobalamin does not pass into the starch granules, although it is known to be soluble in a medium of similar composition (Table 3). It seems probable that it does not dissolve in the hydrated granules because it cannot penetrate their structure. The behaviour of other large molecules may be explained in the same way, and since substances of intermediate size (e.g. hexaose) separate from both the larger (amylose) and smaller (glucose) molecules it is clear that they must penetrate to an intermediate extent, and that there is no definite pore size which separates materials into those which are larger and those smaller, as considered by Lindqvist & Storgårds (1955). Rather, molecules must be considered as varying in the depth to which they penetrate the starch grains. From the fact that, with few exceptions, molecules are eluted in the descending order of their mol.wt., it is highly probable that the major factor determining the depth to which molecules penetrate the granules is molecular size. In this view substances of the same size penetrate to the same degree, and assuming no solubility-partition effects, they would be eluted in the same volume. This conception is supported by the similar behaviour of substances of identical mol.wt. The three disaccharides which were examined behaved identically (Fig. 1), and the three monosaccharides were almost the same (two at 49 ml., one at 51 ml.). Maltotriose behaved irregularly as compared with raffinose. Solubility factors may play some part in exceptional cases.

The behaviours of small charged molecules (Table 1) are more complex than those of neutral molecules, and the results do not support Lindqvist & Storgårds (1955) who considered that columns of starch in citrate buffer separated peptides and amino acids in the range of mol.wt. 75–500. It is clear from Table 1 that amino acids and peptides of this size vary in their behaviour, and that the mol.wt. is not the primary factor determining the order of elution. The effect of change of pH, and of salt concentration, suggests that the starch is acting as an ion exchanger. From the data of Table 1 it is evident that acidic substances are not greatly retarded in either acidic or alkaline medium. There is some evidence that the addition of a negative charge may slightly accelerate the elution of a molecule [compare: galacturonic acid (13·0 ml.) and glucose (14·0 ml.) at pH 8·5, Table 1; glucuronic acid (12·0 ml.), galacturonic acid (12·5 ml.), glucose (14·0 ml.) and glucuronolactone (15 ml.) in 0·05M phosphate buffer at pH 7·0]. Bases tend to be retarded (see glucose and glucosamine, also streptomycin, thiamine, riboflavin, Table 1) and this effect is greatest in an acidic medium and minimized by the addition of salt. Aromatic character may also produce retardation (see benzoic acid and aniline, tyrosine, tryptophan, folic acid) but, unlike that due to basic character, the aromatic retardation is not reversed by increasing the salt concentration.

Among the amino acids, the dicarboxylic acids move most rapidly, and after them are the 'normal' monocarboxylic mono-amino acids. Cystine, threonine, methionine and serine are all retarded a little, and after them the basic amino acids. The extent of separation of glycine and alanine is of the same order as Moore & Stein (1948) observed on a larger scale. Columns of starch in buffer solutions could be used for the separation of many amino acids, especially the aromatic and basic ones. Their capacity would probably not be high, perhaps only one-tenth of a conventional ion-exchange resin, if judged by the acid-binding capacity of starch (Radley, 1953).

On columns of untreated potato starch most proteins do not penetrate the starch grains and accordingly they move with the leading edge of the solvent (Table 2) when they are not adsorbed. The upper limit of usefulness of such columns is in the region of mol.wt. 1000. When the starch grains are

swollen they are made penetrable to larger molecules and considerable resolution can be obtained, though there is much overlapping among molecules larger than haemoglobin (mol.wt. 67 000). The separation of large molecules can be increased when starch which has been more extensively swollen is used, but the slowness of the columns is a disadvantage. On columns of starch swollen 4·5 times the distance between the peaks of albumin and globulin is increased from 1 ml. (on threefold-swollen starch) to 4·0 ml. The lower range in which threefold-swollen starch is useful appears to be about 1300.

The greater permeability of heat-treated starch is associated with an increased tendency for large bases to be adsorbed, probably because these are adsorbed only after they penetrate the grains. Thus protamin which is not retarded on unswollen starch in the presence of salt (Table 2) cannot be eluted from swollen starch in salt solution. Cytochrome *c* is markedly retarded on swollen starch though salt releases it from adsorption (Table 3). Polymyxin B, which is small enough to penetrate untreated starch to some extent, is considerably retarded by it (Table 2). These observations suggest that swollen starch may be a suitable material to use as an ion exchanger in the separation of basic proteins, and possibly other substances. The adsorption of most proteins appears to be negligible in the presence of 0·25 M sodium chloride. This concentration is ten times that required to free cytochrome *c* from swollen starch (Table 3).

Insulin was eluted in the same position as inulin, which has a mol.wt. of about 5700 (Hirst, McGilvray & Percival, 1950). This value is about half of the usually accepted figure (12 000; Edsall, 1953), although it is in agreement with the value which Harfenist & Craig (1952) obtained by countercurrent distribution of the dinitrophenyl derivatives of insulin.

Soluble laminarin (Fig. 2) behaved as if it were larger than inulin. Connell, Hirst & Percival (1950) estimated its mol.wt. at about 3600, but conceded the possibility of it being larger.

It is desirable that a larger number of proteins should be examined on starch columns to establish the limitations of this method for determining mol.wt. In particular, the effect of hydration of molecules, and of divergence from spherical shape, may alter the behaviour, the former, presumably, affecting a more rapid elution and the latter producing a retardation. These factors may account for some of the discrepancies which have been noted, e.g. carbonic anhydrase. Mol.wt. has been considered here merely as a convenient approximation of molecular size, for substances composed largely of carbon, oxygen and nitrogen.

For general use in the laboratory it would be valuable to have a number of 'markers' of known molecular size, shape and weight, for comparison with substances of unknown dimensions on starch columns. Various polysaccharides might serve this purpose, or possibly synthetic polymers. Should these become available, chromatography on starch may provide a simple method of determining the molecular size of a wide range of proteins and polysaccharides. The method has the advantage that measurements can be made in mixtures, provided that the substance under examination can be estimated by some specific method, such as enzyme activity.

Table 6. *Comparison of starch and cellulose columns*

The data have been calculated per 10 ml. of mobile phase, as indicated by colloidal carbon on starch columns, and haemoglobin on cellulose. The volume of stationary phase ($S$) for urea, in column 5, was calculated from the observed $R$ value of urea (relative to carbon or haemoglobin) and the volume of mobile phase ($M$), using the formula $R=(M/S)/(1+M/S)$. It was assumed that the concentrations of urea in the mobile and stationary phases were equal.

| Column | Wt. of dry support (g.) | Vol. of hydrated support (ml.) | Water in hydrated support (ml.) | Calc. stationary phase for urea (ml.) |
|---|---|---|---|---|
| Untreated potato starch | 19·8 | 21·5 | 9·3 | 13·0 |
| Swollen maize starch | 4·95 | 20·4 | 19·0 | 15·6 |
| Cellulose | 3·7 | 2·7 | 1·31 | 0·75 |

Three proteins were eluted in volumes which were inconsistent with the reported mol.wt. Carbonic anhydrase appeared in a volume appropriate to a mol.wt. of about 40 000 rather than 30 000 (Edsall, 1953). Further study with larger columns is necessary, since the differences were not large.

The elution of ox myoglobin in a position between $\beta$-lactoglobulin (mol.wt. 38 000) and pepsin (35 000) suggested that its mol.wt. is double the usually accepted value of 17 000 (Edsall, 1953) for horse myoglobin.

Cellulose columns showed little capacity to separate substances of different mol.wt. Haemoglobin and urea were eluted almost together. The differences between the two types of starch

columns and the cellulose column are clearly seen when the weight of dry supporting material, the volumes of stationary hydrated support and the amount of the water in it are compared, a standard volume (10 ml.) of mobile phase being taken as a basis (Table 6). The volume of hydrated support in the starch columns was twice that of the mobile phase, but with cellulose the hydrated support was one-third of the mobile phase. The proportion of the hydrated support, and of the contained water, which was available for solution of urea was much smaller in cellulose columns than in starch columns.

Thus the capacity to separate materials on a column, by a partition based on varying degrees of penetration, appears to be unique for starch, and probably depends on the structure of the hydrated granules. A possible explanation, for which there is some microscopical evidence (Frey-Wyssling, 1953), is that the amylopectin chains radiate from the centre of the starch granule, and the distance between neighbouring chains is very small at the centre and increases toward the periphery. Solute molecules may penetrate only to the depth at which their diameters are equal to the distance between amylopectin chains. As a result the volume of stationary phase in which solutes become distributed is determined by their molecular size.

## SUMMARY

1. From columns of potato starch in buffer neutral molecules were eluted in the descending order of their molecular weights. There was good resolution of substances in the molecular weight range of 100 to 1000.

2. The behaviour of small charged molecules was complicated by ion-exchange effects, which could be minimized by the addition of salt.

3. Large molecules remained outside untreated starch grains. Starch which has been swollen by warming in water was permeable to larger molecules, and in columns separated them in the range of molecular weights 150 000 to 1300.

4. The possibility of determining molecular weights with these columns is discussed. Values of 6000 for insulin and 35 000 for ox myoglobin were obtained.

5. It is suggested that columns of starch in water form a new type of partition system in which the volume of the stationary phase is determined for each substance by the depth to which it can penetrate the starch granules.

The authors wish to express their thanks to Dr W. J. Whelan and Dr R. L. M. Synge for helpful discussions, and also to those who provided materials.

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# The Use of *p*-Chloromercuribenzoic Acid in the Determination of Ascorbic Acid with 2:6-Dichlorophenolindophenol

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(*Received* 8 *September* 1955)

Methods based on the ability of ascorbic acid to reduce the dye 2:6-dichlorophenolindophenol (indophenol) to a colourless compound (Tillmans, Hirsch & Reinshagen, 1928) have been widely used in the estimation of ascorbic acid. Many other substances, including thiol compounds, thiosulphates, hydrogen sulphide, polyphenols and reductones (Lugg, 1942; Harris & Mapson, 1947) also decolorize indophenol and may therefore interfere with the determination of ascorbic acid. Most of these interfering substances reduce indophenol more slowly than does ascorbic acid (Harris & Mapson, 1947). The presence of substances which act in this way has been demonstrated in whole serum (Bierring, Mathiessen & Nielsen, 1945), and also in extracts of various mammalian tissues, e.g. kidney (Hopkins & Slater, 1935), brain (Young & Mitolo, 1934), whole blood (Butler & Cushman, 1940) and urine (Evelyn, Malloy & Rosen, 1938; Scarborough & Stewart, 1937). Mann & Leone (1953) reported that about 85% of the indophenol-reducing activity of hog semen was due to the presence of ergothioneine.

To increase the specificity of the indophenol method for the estimation of ascorbic acid Van Eekelen & Emmerie (1936) proposed the use of mercuric acetate to precipitate some of the interfering substances. An alternative method, based on the formation of complexes of interfering substances and formaldehyde, was proposed by Lugg (1942). However, methods based on these principles have certain disadvantages, which are discussed below.

In an attempt to devise a specific yet simple method for the determination of ascorbic acid in mammalian tissues we have found that *p*-chloromercuribenzoic acid successfully suppresses the interference due to thiol compounds. The results obtained by using this reagent in the determination of ascorbic acid in human plasma, erythrocytes and urine are presented. Preliminary communications of some of the results have already been given (Owen, Iggo & Horn, 1954; Owen & Iggo, 1955).

## MATERIALS AND METHODS

*Reagents.* L-Dehydroascorbic acid was prepared by oxidation of ascorbic acid with quinone (Paterson, 1950). A stock solution of the sodium salt of 2:6-dichlorophenolindophenol (indophenol) containing 100 mg./100 ml. was made up weekly and kept at 5°. A stock solution [20% (w/v)] of metaphosphoric acid was made up at intervals of not longer than 4 days and kept at 5°. *p*-Chloromercuribenzoic acid (CMB) was prepared according to the method of Whitmore & Woodward (1946) and a solution containing 200 mg./100 ml. in 0·05N-NaOH was used. A solution of homocysteine was prepared by shaking for 30 min. 50 mg. of zinc dust in 20 ml. of a solution of homocysteine (L. Light and Co.), 68 mg./100 ml., in 2% (w/v) metaphosphoric acid and filtering off the excess of zinc. Titration against standard iodine solution showed that reduction was 90% complete. A solution of hydrogen sulphide was prepared by bubbling $H_2S$, generated by warming $Sb_2S_3$ in conc. HCl, into 2% (w/v) metaphosphoric acid. The concentration of hydrogen sulphide was determined by titration against $KMnO_4$.

Adsorption reagents: aluminium oxide for chromatographic analysis. Floridin earth, fuller's earth, light kaolin and acid-washed kieselguhr (all from British Drug Houses, Ltd.), Florisil (Floridin Co., Warren, Pa., U.S.A.), Celite (Johns Manville and Co. Ltd., London). Deactivated charcoal was prepared according to the method of Dalgliesh (1955).

*Plasma.* Protein-free filtrates of human plasma (oxalated or heparinized) were prepared by adding 2 vol. of plasma to 3 vol. of 3% (w/v) metaphosphoric acid.

*Erythrocytes.* Protein-free filtrates of human erythrocytes were prepared after preliminary treatment with carbon monoxide (Butler & Cushman, 1940). Whole blood (20 ml.) (oxalated or heparinized) was centrifuged and the leucocyte layer removed. The erythrocytes, resuspended in the plasma, were then placed in a tonometer (500 ml.), into which was led a steady stream of carbon monoxide generated from sodium formate and conc. $H_2SO_4$. The tonometer was rotated for 15 min. After centrifuging, 2 vol. of erythrocytes was added without delay to 3 vol. of 6% (w/v) metaphosphoric acid.

*Urine.* Fresh human urine, after filtration, was diluted to give a suitable indophenol-reducing activity. To 2 vol. of diluted urine was added 3 vol. of 3% (w/v) metaphosphoric acid.

*Rat liver and kidney.* The animals were killed by dislocation of the cervical vertebrae, and the organs dissected out and freed from any adhering fat without delay. Portions of the liver (approx. 3 g.) or the whole kidney (approx. 2 g.) were comminuted in 10 ml. of 6% (w/v) metaphosphoric acid in an all-glass tissue homogenizer. The extracts, made up to 15 ml., were allowed to stand for 15 min. and then filtered through two layers of Whatman no. 42 filter paper.

*Measurement of* pH. A Marconi pH meter (type TF S11D) was used.

*Measurement of indophenol-reducing activity.* The method used was similar to that of Evelyn *et al.* (1938). Into a

10 mm. cuvette was pipetted 2·0 ml. of the test solution followed by 0·5 ml. of sodium citrate (concentration according to final pH required) and 1·0 ml. of indophenol. After rapid mixing the optical density of the solution was measured with a Unicam SP. 600 spectrophotometer at 520 mμ. at 30 sec., or stated intervals, after the addition of indophenol. Allowance was made for any turbidity or colour in tissue extracts by measuring the optical density of the solution immediately after decolorization of the remaining indophenol with a slight excess of ascorbic acid. Calibration curves were prepared for each experiment with standard solutions of ascorbic acid in 1·8% (w/v) metaphosphoric acid buffered to the approximate pH.

*Reaction with* p-*chloromercuribenzoic acid* (*CMB*). To 3 vol. of the test solution was added 1 vol. of CMB. After standing for 5–10 min. a precipitate, consisting of excess of reagent together with any insoluble complex formed by interaction with substances present, was removed by centrifuging and the supernatant solution used for colorimetry. CMB is only sparingly soluble in aqueous solutions at pH 2–5, and the concentration of CMB in the supernatant was not determined.

## RESULTS

The stability of indophenol at low pH was examined by measuring the rate of decrease in optical density of dye in metaphosphoric acid solutions buffered with sodium citrate (Fig. 1). Below pH 3·5 appreciable fading occurred within 5 min. of the addition of the dye. The rate of fading was that of a first-order reaction. At pH 5·0 the solution was purple, indicating that some of the dye was in the anionic form.

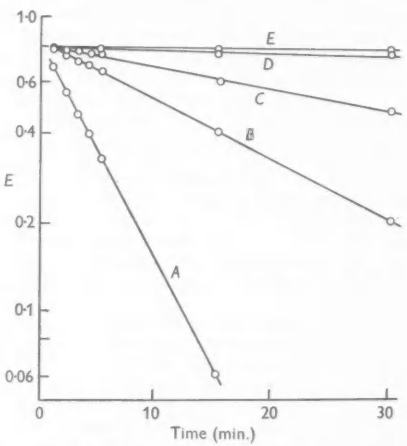


Fig. 1. Effect of pH on the stability of indophenol. Each solution comprises 2·0 ml. of 2% (w/v) metaphosphoric acid buffered with sodium citrate + 1·0 ml. of indophenol (10 mg./100 ml.). pH: *A*, 2·0; *B*, 2·4; *C*, 2·9; *D*, 3·5; *E*, 4·2.

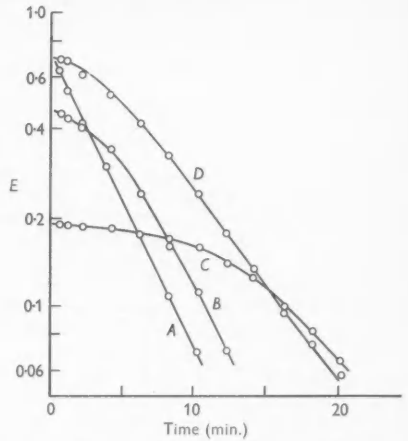


Fig. 2. Effect of added substances on the stability of indophenol at pH 1·8. *A*, 2·0 ml. of 2% (w/v) metaphosphoric acid + 0·5 ml. of $H_2O$ + 1·0 ml. of indophenol (10 mg./100 ml.); *B*, as *A* but with metaphosphoric acid containing ascorbic acid, 1 mg./100 ml.; *C*, as *A* but with metaphosphoric acid containing ascorbic acid, 2 mg./100 ml.; *D*, as *A* but with 0·5 ml. of reduced dye solution (125μg. of reduced dye) instead of 0·5 ml. of $H_2O$.

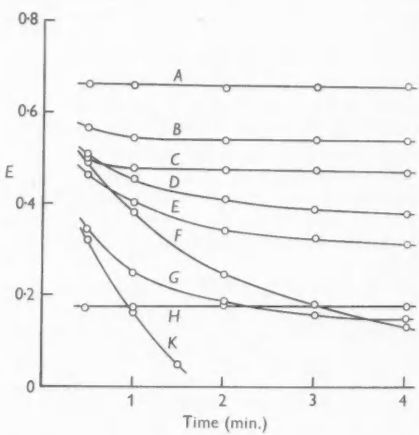


Fig. 3. Rates of reaction of various substances with indophenol at pH 4·5. Each solution comprises 2·0 ml. of 2% (w/v) metaphosphoric acid + 0·5 ml. of 12% (w/v) sodium citrate + 1·0 ml. of indophenol (10 mg./100 ml.) with the following additions: *A*, none; *B*, thiourea, $5 \times 10^{-4}$M; *C*, $Na_2S_2O_3$, $0{\cdot}6 \times 10^{-4}$M; *D*, $H_2S$, $5 \times 10^{-4}$M; *E*, $Na_2SO_3$, $2{\cdot}5 \times 10^{-4}$M; *F*, glutathione, $5 \times 10^{-4}$M; *G*, thioglycollic acid, $2 \times 10^{-4}$M; *H*, ascorbic acid, $1 \times 10^{-4}$M; *K*, cysteine, $5 \times 10^{-4}$M.

Partial decolorization of the dye with ascorbic acid leads to a decrease in the rate of fading which occurs spontaneously at pH about 2·0 (Mindlin & Butler, 1937–8; Lugg, 1942; Stewart, Horn & Robson, 1953). Mindlin & Butler reported, further, that the addition of dehydroascorbic acid did not affect the rate of fading, whereas the addition of reduced (leuco) dye stabilized the dye. We have found, however, that the stabilization of residual dye after the addition of ascorbic acid is incomplete and temporary (Fig. 2). Addition of reduced dye prepared by reaction of dye with zinc dust, or with ascorbic acid, also increased the stability of the dye temporarily. Dehydroascorbic acid had no stabilizing effect.

The reaction of various substances with indophenol was examined by measuring the rate of decolorization of the dye at pH 3·5 and 4·5. Ascorbic acid, thiosulphate and thiourea reacted relatively quickly, so that an equilibrium was reached within 60 sec. (Fig. 3). With the other substances tested an equilibrium was not reached within 4 min. Of the compounds examined all reacted with indophenol more rapidly at the higher pH except thiosulphate, thiourea and ergothioneine, which reduced indophenol more rapidly at the lower pH (Table 1).

CMB did not affect the ability of ascorbic acid to reduce indophenol, but inhibited almost completely the indophenol-reducing activity of all the other compounds tested with the exception of sulphite. This inhibition persisted for at least 5 min. (Table 1). It was found, further, that CMB inhibited the indophenol-reducing activity of glutathione, cysteine, hydrogen sulphide or thiosulphate, when added to metaphosphoric acid extracts of human plasma, erythrocytes, urine or of rat liver, but it did not affect the recovery of ascorbic acid added to these extracts (Table 2).

The effect of CMB on the indophenol-reducing activity (apparent ascorbic acid content) of human plasma, erythrocytes and urine was then

Table 1. *Indophenol-reducing activity of various compounds*

Results are expressed in terms of equivalent ascorbic acid concentration ($10^{-5}$ M).

| | | No addition | | | | | | With CMB (50 mg./100 ml.) |
|---|---|---|---|---|---|---|---|---|
| | | 30 sec. | | 1 min. | | 5 min. | | 5 min. |
| Compound | Concn. ($10^{-5}$ M) | pH 4·5 | pH 3·5 | pH 4·5 | pH 3·5 | pH 4·5 | pH 3·5 | pH 3·5 |
| Ascorbic acid | 5 | 5·0 | 5·0 | 5·0 | 5·0 | 5·0 | 5·0 | 5·0 |
| Sodium thiosulphate | 6 | 3·6 | 4·1 | 3·6 | 4·0 | 4·1 | 3·6 | 0·0 |
| Sodium sulphite | 25 | 5·0 | 4·8 | 5·5 | 5·5 | 8·4 | 7·7 | 2·7 |
| Cysteine | 50 | 7·1 | 2·6 | 10·3 | 6·8 | >20 | >20 | 0·0 |
| 2:3-Dimercaptopropanol | 50 | 6·8 | 5·1 | 9·8 | 7·5 | 13·5 | 11·5 | 0·1 |
| Ergothioneine | 50 | 0·0 | 2·7 | 0·0 | 4·2 | 0·0 | 9·6 | 0·0 |
| Glutathione | 50 | 3·3 | 0·7 | 5·8 | 1·3 | 11·7 | 6·3 | 0·0 |
| Hydrogen sulphide | 50 | 3·1 | 2·6 | 4·4 | 3·2 | 6·1 | 4·8 | 0·2 |
| Homocysteine | 50 | 0·7 | 0·3 | 1·3 | 0·4 | 6·4 | 1·6 | 0·1 |
| Thiourea | 150 | 2·0 | 3·2 | 2·5 | 3·6 | 2·5 | 7·0 | 0·0 |

Table 2. *Effect of* p*-chloromercuribenzoic acid on the indophenol-reducing activity of compounds added to biological material*

Substances were added in amounts calculated to give approximately equal increments in indophenol-reducing activity. Results in terms of ascorbic acid (μg./100 ml.). Values in parentheses give percentage of added ascorbic acid recovered.

| | Addition | | | | | |
|---|---|---|---|---|---|---|
| Material | None | Ascorbic acid (400 μg./100 ml.) | Glutathione (12 mg./100 ml.) | Cysteine (5 mg./100 ml.) | $H_2S$ (1·4 mg./100 ml.) | Thiosulphate (400 μg./100 ml.) |
| Plasma filtrates | 444 | 868 (105) | 448 | 448 | 444 | 448 |
| | 404 | 832 (107) | 404 | 424 | 408 | 416 |
| | 424 | 840 (104) | 448 | 448 | 424 | 416 |
| Erythrocyte filtrates | 232 | — | 240 | 244 | 252 | 232 |
| | 212 | 636 (106) | 232 | 196 | 200 | 216 |
| | 312 | 684 (93) | 308 | — | 288 | 300 |
| Diluted urine | 316 | 688 (93) | 308 | 316 | 300 | 308 |
| | 400 | 796 (99) | 400 | 400 | 400 | 432 |
| | 272 | 664 (98) | 264 | 232 | 256 | 256 |
| Diluted liver filtrate | 452 | 872 (105) | 452 | 452 | 452 | 456 |

examined (Fig. 4). Added to filtered metaphosphoric acid extracts of plasma this compound produced a negligible effect on the apparent ascorbic acid content in filtered extracts of plasma. In contrast to extracts of plasma, metaphosphoric acid extracts of erythrocytes contained appreciable quantities of slow-reacting material. The effect of this was noticeably greater at pH 4·5 than at pH 3·5. The addition of CMB produced a decrease in the apparent ascorbic acid content and progressive decolorization of the dye no longer occurred.

Human urine also showed the presence of slow-reacting substances (Fig. 4). The effect of these, however, was only partly abolished by CMB. Further treatment of the extracts with the adsorption reagents alumina, Celite, Floridin earth, Florisil, fuller's earth, kaolin and kieselguhr failed to remove all the remaining slow-reacting material. Deactivated charcoal removed most of the interfering substances, but at the same time destroyed most of the ascorbic acid.

The quantitative effect of CMB is shown in Table 3. The mean decrease in the apparent ascorbic acid content of plasma was about 2 %, of erythrocytes about 50 %, and of urine about 15 %. In these analyses the reduction of indophenol was measured at pH within the range 3·6–4·4.

In a few experiments the indophenol-reducing activity of filtered metaphosphoric acid extracts of rat liver and kidney was examined. Filtrates of both these tissues contained appreciable quantities of interfering material (Fig. 5), the effect of which was removed almost completely by CMB.

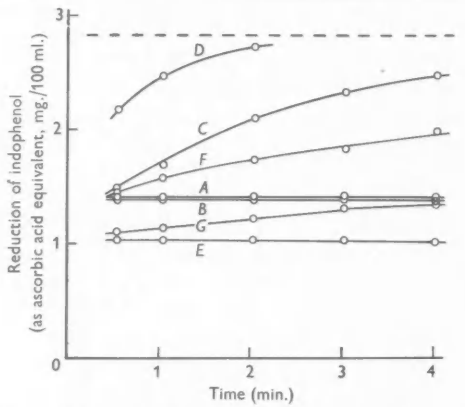


Fig. 4. Effect of *p*-chloromercuribenzoic acid (CMB) on the indophenol-reducing activity of human plasma, erythrocytes and urine. Each solution comprises 2·0 ml. of test solution + 0·5 ml. of sodium citrate + 1·0 ml. of indophenol (4 mg./100 ml.). Test solutions (pH values are those of reaction mixture): *A*, plasma filtrates, pH 3·5; *B*, as *A* + CMB; *C*, erythrocyte filtrates, pH 3·5; *D*, as *C*, pH 4·5; *E*, as *C* + CMB; *F*, diluted urine, pH 3·5; *G*, as *F* + CMB. Amount of CMB added, 50 mg./100 ml. Interrupted horizontal line corresponds to complete decolorization of dye.

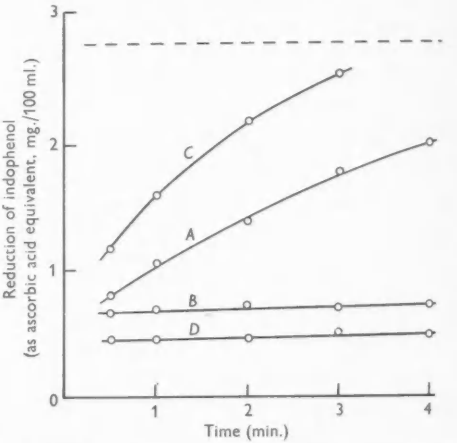


Fig. 5. Effect of *p*-chloromercuribenzoic acid (CMB) on indophenol-reducing activity of rat liver and kidney. Each solution comprises 2·0 ml. of test solution + 1·0 ml. of indophenol (4 mg./100 ml.). Test solutions: *A*, liver filtrate, diluted 1/4, buffered with sodium citrate (≡ 7·5 g. of wet tissue/100 ml.); *B*, as *A* + CMB; *C*, kidney filtrate buffered with sodium citrate (≡ 20 g. of wet tissue/100 ml.); *D*, as *C* + CMB. Amount of CMB added, 50 mg./100 ml. Interrupted horizontal line corresponds to complete decolorization of dye.

Table 3. *Effect of CMB on apparent ascorbic acid content of plasma, erythrocytes and urine*

Results are given as means (with ranges in parentheses) in terms of ascorbic acid (mg./100 ml.). The amount of CMB added was 50 mg./100 ml.

| Material | No. of expts. | Without CMB | With CMB | Differences |
|---|---|---|---|---|
| Plasma | 40 | 0·56 (0·09 to 1·40) | 0·55 (0·10 to 1·40) | 0·01 (−0·09 to +0·07) |
| Erythrocytes | 12 | 0·47 (0·21 to 1·39) | 0·25 (0·10 to 0·94) | 0·22 (+0·01 to +0·45) |
| Urine (diluted) | 12 | 0·77 (0·19 to 1·92) | 0·65 (0·02 to 1·79) | 0·12 (0·00 to +0·26) |

## DISCUSSION

To increase the specificity of the indophenol method for the determination of ascorbic acid in biological material, Van Eekelen & Emmerie (1936) proposed treatment of extracts with mercuric acetate to remove thiol compounds and other interfering substances. Subsequently, the extracts were treated with hydrogen sulphide to remove the excess of mercuric acetate and to reduce any dehydroascorbic acid formed by the reaction of ascorbic acid with $Hg^{2+}$ ions. This procedure, however, does not permit the determination of ascorbic acid in the presence of dehydroascorbic acid. Furthermore, several workers have commented on the difficulty of removing, from the extracts, all traces of hydrogen sulphide before measurement of the indophenol-reducing activity, and have drawn attention to the possibility that treatment of tissue extracts with hydrogen sulphide may produce interfering substances not present originally (Johnson, 1933; Mack & Tresler, 1937; King, 1936; Kellie & Zilva, 1936; Bessey, 1938). This has been shown to occur when certain aldehydes, ketones or quinones are treated with hydrogen sulphide (Lugg, 1942; King, 1941; Smythe & King, 1942; Hochberg, Melnick & Oser, 1943). In this connexion Kellie & Zilva (1936) concluded from analyses of ultraviolet absorption spectra that the increase in the indophenol-reducing activity of filtrates of guinea-pig plasma treated with hydrogen sulphide was due to the formation of reducing substances other than ascorbic acid. The presence of increased amounts of interfering substances has also been reported after treatment with hydrogen sulphide of whole-blood filtrates (Butler & Cushman, 1940), and of urine (Evelyn *et al.* 1938; Scarborough & Stewart, 1937).

In contrast, the proposed use of *p*-chloromercuribenzoic acid avoids the errors which may be introduced by the use of hydrogen sulphide and also permits the estimation of ascorbic acid in the presence of dehydroascorbic acid. The action of CMB is presumably due to its powerful tendency to form complexes with thiol groups (Barron & Singer, 1945) so that the reducing ability of the latter is suppressed. However, our findings indicate that it reacts also with thiosulphate and to some extent with sulphite. The nature of the latter two reactions is, at present, not clear.

In view of the relative rapidity of the reduction of indophenol by ascorbic acid, measurement of the amount of indophenol reduced at intervals and extrapolation of these values, plotted against time, to zero time, have been proposed as a means of obviating interference due to slow-reacting substances (Evelyn *et al.* 1938). It is obvious from Fig. 3, however, that for most of the interfering substances a curvilinear extrapolation would be necessary to give zero indophenol reduction at zero time. The inherent difficulties in curvilinear extrapolation make this procedure impracticable.

As a new approach to the problem of non-specific reduction of indophenol Lugg (1942) proposed the use of formaldehyde which condenses to an extent depending on the pH with many interfering substances, and also with ascorbic acid, to form feebly reducing or non-reducing complexes. Of these substances which have been examined only ascorbic acid, reductones and a hydroxytetronic acid (the lactone of 1:2(?):3-trihydroxyprop-1-ene-1-carboxylic acid) condense slowly at pH 1·5 and rapidly at pH 3·5. Thus in the absence of the last-named compounds the difference in indophenol-reducing activities after condensation at pH 1·5 and 3·5 respectively may be taken as a measure of the ascorbic acid present. Methods based on this principle have been applied to the estimation of ascorbic acid in urine (Mapson, 1953; McSwiney, Clayton & Prunty, 1954). However, this procedure is laborious (Harris & Mapson, 1947) and requires careful control of conditions (Snow & Zilva, 1944). We have found, in agreement with others (McSwiney *et al.* 1954), that this procedure cannot accurately be applied to materials in which the concentration of ascorbic acid is low, i.e. less than 1·0 mg./100 ml. Nevertheless, in normal urine, where the concentration of ascorbic acid is relatively high, the formaldehyde method may provide a more accurate result than that given by the method described in this paper, since the result obtained by the latter applied to urine includes some reducing material other than ascorbic acid.

The negligible effect of CMB on the indophenol-reducing activity of plasma is consistent with the absence from plasma extracts of substances reacting slowly with indophenol. The interfering material in human erythrocytes presumably includes glutathione and ergothioneine. The absence of progressive decolorization of indophenol after the addition of CMB to extracts of plasma or erythrocytes suggests that the results represent the true ascorbic acid content, though the presence of substances other than ascorbic acid, which react rapidly with indophenol and which are not removed by CMB, is not excluded. Examination of the reduction of indophenol by these filtrates by the 'continuous-flow' method devised by Harris & Mapson (1947) might enable this point to be settled.

Using formaldehyde, Mapson (1953) showed the presence of two groups of interfering substances in urine. The first group contains substances which form non-reducing complexes rapidly at pH 0·6; at this pH ascorbic acid condenses only slowly.

Any thiol compounds, sulphides and thiosulphates present are included in this group, and it seems reasonable to conclude that this group corresponds roughly to the material removed by CMB. The interfering substances in the second group condense only slowly both at pH 0·6 and at pH 3·5, whereas ascorbic acid itself condenses rapidly at this latter pH. It is concluded that the interfering material remaining after treatment of urine extracts with CMB corresponds roughly to this second group of substances and also to the interfering material not removed by treatment with mercuric acetate (Evelyn *et al.* 1938). The nature of this material remains unknown. It will include any polyphenols present (Lugg, 1942).

The instability of indophenol at low pH is characteristic of indophenol dyes (Karrer, 1950), the latter decomposing to give quinones and aminophenols. The partial stabilization of indophenol by reduced (leuco) dye may be due to the formation of a reversible complex of oxidized and reduced dye, possibly analogous to quinhydrone. The stabilization, however, is not sufficient to enable the reduction of indophenol to be measured satisfactorily at pH 3·0, as has been advocated (Bessey, 1938; Harris & Olliver, 1942) to lessen interference due to other reducing substances. Indeed, the interference due to some compounds, e.g. thiosulphate and ergothioneine, is greater at lower pH.

## SUMMARY

1. The stability of indophenol at low pH has been examined. The amount of spontaneous fading which occurs below pH 3·5 makes the colorimetric estimation of indophenol-reducing activity impracticable in solutions of pH less than this value.

2. The reaction of various reducing substances with indophenol and the interference which their presence would cause in the estimation of ascorbic acid has been examined.

3. Added *p*-chloromercuribenzoic acid (CMB) does not affect the reaction between ascorbic acid and indophenol, but it suppresses almost completely the decolorization of indophenol by a number of interfering substances.

4. Metaphosphoric extracts of human plasma contain only negligible quantities of interfering material removed by CMB, whereas extracts of human erythrocytes, rat liver and kidney contain appreciable amounts of interfering material which are almost completely removed by this compound. Human urine contains interfering material, some of which is removed by CMB.

5. It is concluded that *p*-chloromercuribenzoic acid may be used to increase the specificity of the indophenol method for the determination of ascorbic acid in biological material.

We are greatly indebted to Dr A. B. Roy for the gift of *p*-chloromercuribenzoic acid and to Dr T. Mann for a gift of ergothioneine. We should like to thank Dr C. P. Stewart for his constant advice and criticism at all stages of the work. The participation of one of us (B.I.) was made possible by a grant from the Scottish Hospital Endowments Research Trust.

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## CORRIGENDA

The separation and quantitative determination of the component sugars of mucoproteins

By A. Gottschalk and G. L. Ada

Volume 62 (1956)

p. 681, col. 1, line 22:

*delete* carbazole (Dische, Shettles & Osnos, 1949)

p. 681, col. 1, line 31:

*for* Dische (1947) *read* Dische, Shettles & Osnos (1949)

# The Separation and Quantitative Determination of the Component Sugars of Mucoproteins

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(*Received* 20 *July* 1955)

In recent years increasing attention has been given to a class of carbohydrate–protein complexes, largely because of the biological activity associated with them (gonadotrophin, thyroglobulin, blood-group substances, inhibitors of influenza-virus haemagglutination, Castle's intrinsic factor, etc.). In many of these the carbohydrate moiety represents analytically a minor portion only, though, as in virus inhibitors, this minor portion carries the functional group (Gottschalk, 1954). This subclass of complexes, in which protein predominates, may for convenience be referred to as mucoproteins.

In any quantitative analysis of the carbohydrate moiety of mucoproteins three major difficulties arise: (1) in most cases, if not all, the carbohydrate is a mixed polysaccharide; (2) pure samples of the material to be analysed are usually available in small amounts only; (3) the amino acids liberated on hydrolysis of the mucoprotein are known to interact with sugars. Though colorimetric methods, such as the orcinol (Tillmans & Philippi, 1929), carbazole (Dische, Shettles & Osnos, 1949) and anthrone (Dreywood, 1946) methods, are available for the overall estimate of the non-amino sugars of mucoproteins, there is a demand for a method allowing the quantitative determination of the individual sugars in mucoproteins.

The application to the intact mucoprotein of the orcinol, carbazole and cysteine reagents by Sörensen & Haugaard (1933), Gurin & Hood (1939) and Dische (1947) respectively for the quantitative determination of the component sugars derives from the observation that the absorption curve of the coloured compound formed varies with the individual sugar. With increasing complexity of the mixture, however, an unequivocal interpretation of the curves becomes more and more difficult (Meyer, 1938; Gurin & Hood, 1939).

An alternative method would be to hydrolyse the mucoprotein gently, to separate the liberated sugars from the amino acids and to identify and estimate the individual sugars by chromatography. This technique was first applied to the quantitative analysis of the carbohydrate of a mucoprotein prepared from human urine (Gottschalk, 1952*a*, *b*). The method has been further developed; the efficiency of each step has been tested and, as described below, in satisfactory operation in this Laboratory for some time. With this method sugar added to a protein is recovered almost quantitatively; the intrinsic sugar of a mucoprotein is recovered to an extent of 70–90% of that estimated by an independent technique to be present in the original material; the degree of recovery depends upon the type of mucoprotein. As early as 1948, Flood, Hirst & Jones (1948) applied paper partition chromatography to the quantitative analysis of the component sugars of polysaccharides. Since the material analysed by the authors did not contain protein, the main difficulty—the interference of amino acids—did not arise. Glegg, Eidinger & Leblond (1953) and Dixon (1955) have used ion-exchange resins to hydrolyse mucoproteins and simultaneously to separate, by adsorption to the resin, the amino acids from the sugars, which in turn are analysed chromatographically. Even with a soluble mucoprotein (ovomucoid) the recovery in the neutralized hydrolysate was only 61%, and further losses occurred in the later stages, the sugar in the original and after processing being checked by the same method (Dixon, 1955). For insoluble mucoproteins (reticular fibres) no attempt has been made to estimate the recovery (Glegg *et al.* 1953).

## EXPERIMENTAL

### *Materials*

*Mucoproteins.* Bovine serum albumin was a preparation obtained from Armour Laboratories, Chicago. This was used to imitate the protein moiety of a mucoprotein in experiments designed to test the recovery of known amounts of sugars in artificial sugar–protein mixtures. Though bovine serum albumin contains a small amount of intrinsic sugar (0·3%, by orcinol), it was selected for this purpose because it is a well-defined protein. Thyroglobulin was kindly prepared by Professor V. M. Trikojus and Dr M. McQuillan, Melbourne University, according to the procedure of Derrien, Michel & Roche (1948). Ovomucoid was prepared according to Mörner (1894). The homogeneous bovine-submaxillary-gland mucoprotein (BSM) was prepared according to Curtain & Pye (1955). All proteins were stored *in vacuo* and kept for 24 hr. over $P_2O_5$ before use.

*Yeast nucleic acid.* A commercial preparation was purified according to the procedure of Smith & Markham (1950).

*Sugars.* The sugars used were standard preparations, exhibiting with one exception a single spot when tested chromatographically. The sample of chondrosamine contained a small proportion of glucosamine. In experiments with the addition to protein of small amounts of sugar a 0·20% solution of the appropriate sugar was prepared and a portion taken. We are indebted to Professor H. O. Fischer, University of California, Dr H. G. Fletcher, National Institute of Health, Bethesda, Professor W. T. J. Morgan, F.R.S., London, and Dr D. J. Bell, Edinburgh, for generous gifts of rare sugars.

*Ion-exchange resin.* Dowex 50 (200–400 mesh) was prepared for use in the hexosamine determination according to Boas (1953). When applied for the removal of amino acids before the chromatographic analysis of the sugars, it was necessary to wash the regenerated column until the effluent was completely free of acid.

### *Analytical methods*

*Determination of total carbohydrate (non-amino sugars).* The overall recovery of the intrinsic sugars in mucoproteins by the method described was checked by using an independent procedure to determine the total carbohydrate in the original material. Of the various methods available the orcinol method appears to be least susceptible to interference by the protein components (Gurin & Hood, 1939; Dische *et al.* 1949). To test this point further the following mixtures were submitted to the orcinol method: 0·15 mg. of D-glucose + 3·6 mg. of bovine serum albumin, 0·15 mg. of soluble starch + 3·6 mg. of bovine serum albumin, 0·05 mg. of D-glucose + 0·05 mg. of cysteine hydrochloride + 0·05 mg. of DL-tryptophan; each mixture present in 1·0 ml. of water. The procedure described by Hewitt (1937) was used, the colour intensity being measured in a photoelectric colorimeter fitted with a green filter (510 mμ.). The values obtained coincided within the experimental error (±3%) with the sugar controls (correcting for the intrinsic sugar of bovine serum albumin), indicating non-interference by the nitrogenous components tested. The result of total carbohydrate (non-amino sugar) obtained by the orcinol method is referred to as the 'orcinol value'.

It may be mentioned that with thyroglobulin and a mucoprotein to be discussed in a subsequent paper the total carbohydrate was determined both by the orcinol method and the tryptophan method in the modification by Badin, Jackson & Schubert (1953). Though in the two colour reactions the conditions of sugar degradation and the coloured compounds formed differed, the results agreed within 6%. In all these tests the standard was a sugar mixture whose composition was derived from a preliminary chromatographic run of the processed mucoprotein (see below).

*Determination of amino sugars.* All samples were hydrolysed with 2N-HCl in a sealed tube at 100° for 6 hr. A longer treatment than is found necessary to obtain a maximum value should be avoided in view of the slight destruction of hexosamine under the impact of hydrochloric acid (Blix, 1948; Strange & Powell, 1954). For quantitative determination of free hexosamine the method of Elson & Morgan (1933) was used. On several occasions the liberated hexosamines, before application of the Elson–Morgan procedure, were purified by adsorption to and elution from a cation-exchange resin (Dowex 50) according to Boas (1953). In all cases the values obtained by the two procedures closely agreed. *N*-Acetylhexosamine was estimated according to Morgan & Elson (1934).

*Uronic acids.* The method of Dische (1947) was used to test for the presence of hexuronic acids.

### *Paper partition chromatography*

*General procedure.* The technique and solvents used have been described previously (Gottschalk, 1955*a*). For the detection of hexoses, pentoses and methylpentoses aniline hydrogen phthalate was used (Partridge, 1949). For routine purposes butanol–pyridine–water and butanol–acetic acid–water were preferred as solvents. Amino sugars were located by the Elson–Morgan reagents as applied to chromatography by Partridge (1948). For quantitative evaluation of the individual sugar an appropriate concentration of the sugar mixture was run together with a serial dilution of the standard, each dilution differing by 20% from the previous one. For aldohexoses and methylpentoses concentrations within the range 0·2–0·4% allowed an estimate to be made with an accuracy of ±10%. The optimum concentration range for pentoses was found to be slightly lower. It is essential to bring the concentration of the sugar under investigation within this range, since the sensitivity of the method decreases with increasing concentration. A preliminary run was necessary to provide information about the nature and the approximate concentration of the sugars present. A standardized micropipette was used to deliver 3·5 μl. of the sugar solution to the paper.

When in the course of the procedure it became desirable to check by an independent method the amount of sugar isolated chromatographically as described above, the sugar was eluted from the paper in the following way: an area, 2 in. × 1 in., located by a guiding spot, was cut out of the paper. After both ends had been slightly pointed the paper was mounted on a glass capillary, one point touching the tip of a burette containing water, and the other draining into a test tube. The sugar was eluted by irrigation with 2·5 ml. of water over a period of 2 hr., conditions found to be adequate for quantitative elution of the amount of sugar applied. A paper blank of equal size was similarly treated. Since even elaborate pretreatment of the chromatographic paper did not prevent the continual release of orcinol-reacting material, a quantitative sugar estimate of the eluate could only be made by heavier loading of the paper. Instead of 3·5 μl., up to 40 μl. of the test solution was applied to the paper, thus reducing the value of the blank to about 5% of that of the sugar eluate.

To obtain an estimate of the approximate proportions of glucosamine and galactosamine, if both proved to be present, the purified hexosamines (see above) were chromatographed with collidine (at 4°) or butanol–pyridine–water as solvent. When the concentration of the individual hexosamine in both assay and standards is brought within the range 0·4–0·8%, a satisfactory resolution of the two compounds is obtained. The $R_F$ values of glucosamine and galactosamine in collidine are 0·32 and 0·26 respectively, and in butanol–pyridine–water are 0·21 and 0·17 respectively.

*Identification of sugars.* The component sugar may be identified by chromatography of the unknown and a standard set of sugars in the four solvents described by Gottschalk (1955*a*). When the sugar thus characterized is one commonly found in mucoproteins, this procedure may be regarded as satisfactory for provisional identification.

*Identification of amino sugars.* Following Gardell, Heljkenskjold & Rochnovlund (1950) the hexosamines were oxidized with ninhydrin to the corresponding pentoses, which were identified by chromatographic analysis. It was found desirable to remove the hexosamines from the acid hydrolysate before carrying out the ninhydrin procedure. This was effected by adsorption to and elution from a cation-exchange resin (Boas, 1953).

### *Analysis of mucoproteins*

The essential steps of the procedure to be described are: (1) acid hydrolysis of the mucoprotein; (2) neutralization and concentration of the hydrolysate; (3) removal of unhydrolysed protein moiety and/or polypeptides by ethanol precipitation; (4) removal of amino acids and small peptides by ion-exchange resin; (5) identification and quantitative determination of the individual sugars by paper partition chromatography.

A comparison at different stages of the orcinol value with that of the original material served as a guide for the elaboration of optimum conditions for quantitative sugar recovery before chromatographic separation of the individual sugars. From preliminary experiments it became evident that a major factor interfering with the quantitative recovery from a mucoprotein of the intact monosaccharides is the interaction between the released sugars and amino acids (Maillard, 1912; Gottschalk & Partridge, 1950; Gottschalk, 1952*a*). To reduce to a minimum the rate of this detrimental reaction, the method was so designed that at high temperatures (e.g. step 1) the volume of the reaction mixture was kept reasonably large and that during concentration procedures (e.g. step 2) low temperatures were maintained.

*Step* 1. About 25 mg. of the mucoprotein was digested with 5·0 ml. of 0·25 N-$H_2SO_4$. A period of 18 hr. heating at 100° in a sealed tube proved to be optimum; more severe conditions did not result in a greater yield of monosaccharides.

*Step* 2. The cooled hydrolysate was carefully neutralized to pH 7·0 with N-NaOH, and a drop of acetic acid added to the mixture to safeguard the sugar during the following concentration. This concentration was carried out from the frozen state *in vacuo*, the distilling flask being submerged in an ice bath to prevent any rise of temperature, especially toward the end of the drying process. In this way the mixture was concentrated to approximately 2 ml.

*Step* 3. Ethanol was directly added to the mixture in the distilling flask to give a final concentration of 95% (v/v). The mixture was then transferred quantitatively to a centrifuge cup and allowed to stand in the refrigerator overnight. The precipitate formed was removed and washed on the centrifuge. To the pooled supernatants was added 50 ml. of water containing one drop of acetic acid, and the mixture was concentrated on a steam bath. To facilitate removal of ethanol without decreasing the volume to less than 8 ml., two portions of 10 ml. of water were added during the latter part of the procedure.

*Step* 4. The solution (8 ml.; pH about 4·0) was passed through a well-washed Dowex 50 column and followed by 12 ml. of water. The effluent was concentrated from the frozen state to about 2 ml. as described in step 2. The concentrate was carefully adjusted to pH 5·0 with 0·5 N-$Na_2CO_3$ and evaporated to dryness *in vacuo* at 0°. The absence of any ninhydrin-positive reacting material in the residue indicated complete removal of amino acids.

*Step* 5. The dried sugars were taken up in an appropriate amount of water, and samples taken for sugar determination by the orcinol method and for chromatographic analysis (see Methods). The accuracy of the determination of the individual sugar may be increased by eluting the individual sugars (see Methods) and submitting the eluates to one of the standard methods based on the reducing power or colour formation of monosaccharides. The increase in accuracy will justify the additional time-consuming step in special cases only.

## RESULTS

### *Test and application of the method*

The efficiency of the method, i.e. the degree of recovery of sugar present in the original material as indicated by an independent method, was tested in a twofold way. First, a known amount of sugar(s) was added to a protein and, after processing as above, the result of the chromatographic analysis was compared with the amount of sugar added. As a further check the chromatographically isolated sugar was eluted and the orcinol value of the eluate compared with sugar added. Secondly, mucoproteins were submitted to the procedure described and the chromatographic result was compared with the orcinol value of the original material.

The results of representative analyses are shown in Table 1. As may be seen, the recovery by chromatography of sugars added to a protein is almost quantitative, whether estimated by visual comparison with the standard or by determination of the sugar eluted from the paper. The recovery of intrinsic sugar of a mucoprotein varies between 70 and 90%.

A complete analysis (representative values) of the carbohydrate moiety of the mucoproteins referred to in Table 1, as obtained by the method described, is given in Table 2. Mention may be made that no hexuronic acid was found in the mucoproteins investigated.

### *Determination of ribose in purine nucleotides*

For comparison a sugar linked to nitrogenous material other than amino acids was determined chromatographically. Purified yeast nucleic acid (0·20 mg.) was digested with 0·25 ml. of N-$H_2SO_4$ for 1 hr. at 100° in a sealed tube. After neutralization with $Ba(OH)_2$ the precipitate was removed and washed and the pooled supernatants were dried *in vacuo* at 20°. The residue was extracted with hot ethanol, the extract evaporated to a small volume and chromatographed with appropriate ribose standards. The yield of ribose, as free sugar, was 40 $\mu$g. Another 0·20 mg. of nucleic acid was similarly digested, and the percentage of purine bases

Table 1. *Recovery by the chromatographic and orcinol methods of sugar from sugar–protein mixtures and mucoproteins*

| Material and wt. (mg.) | Sugar content of original material (mg.) | Final recovery of sugar: By orcinol (%) | Final recovery of sugar: By chromatography (%) | Final recovery of sugar: Orcinol value of eluate from chromatogram (%) |
|---|---|---|---|---|
| Bovine serum albumin, 25·0 | 0·08* | — | — | — |
| Galactose, 0·55; mannose, 1·10; bovine serum albumin, 25·0 | 1·73 | 92 | 90 | — |
| Glucose, 1·20; bovine serum albumin, 25·0 | 1·28 | 95 | — | 90 |
| Thyroglobulin, 25·0 | 1·20* | 95 | 90 | — |
| Ovomucoid, 23·0 | 1·90* | 82 | 70 | — |
| BSM, 30·0 | 0·68* | 88 | 68 | — |

* Orcinol value (expressed as free monosaccharides).

Table 2. *Analysis of the carbohydrate moiety of some mucoproteins*

Values are expressed as free monosaccharides.

| Mucoprotein | Sugar components*: Galactose (%) | Sugar components*: Mannose (%) | Sugar components*: Fucose (%) | Amino sugars: Total (%) | Amino sugars: Glucosamine (%) | Amino sugars: Galactosamine (%) | Amino sugar / Non-amino sugar† |
|---|---|---|---|---|---|---|---|
| Thyroglobulin‡ | 1·2 | 2·7 | 0·4 | 4·0 | 4·0 | 0 | 0·85 |
| Ovomucoid§ | 1·4 | 4·3 | 0 | 14·1 | 14·1 | 0 | 1·71 |
| BSM | 0·7 | 0·2 | 0·7 | 10·0 | 0·8 | 9·2 | 4·41 |

* Values obtained by chromatography.
† Orcinol value.
‡ See also Ujejski & Glegg (1955).
§ See also Stacey & Woolley (1940, 1942).

present determined by chromatographic analysis according to Smith & Markham (1950). Purine bases found were adenine 18 $\mu$g., guanine 22 $\mu$g., equivalent to 42 $\mu$g. of purine-bound ribose (expressed as free pentose). The chromatographic recovery was therefore 95 %.

## DISCUSSION

A major difficulty in the recovery for analysis of the sugar components of a mucoprotein is the interaction between reducing monosaccharides and amino acids or free amino groups of the protein. Whereas ribose may be easily recovered quantitatively from purine nucleotides without any special precautions (see Experimental), processing of a sugar–protein mixture or complex under the same unrefined conditions resulted in losses up to 80 % of the added or intrinsic sugar. This difficulty has been overcome by maintaining low temperatures where high concentrations of the reactants are unavoidable and by the removal of any nitrogenous material before drying the sugars. Though these precautions necessitated several steps, the recovery of sugar added to a protein was almost quantitative. This is an unequivocal test for the efficiency of the procedure in recovering free sugar.

With mucoproteins the intrinsic sugar was recovered to an extent of 70–90 %, the orcinol value of the original material being taken as 100 %. There is no evidence to suggest that the orcinol method, when applied to intact mucoproteins, gives a value which is appreciably too high, provided that the correct standard as predetermined chromatographically is used. Our experiments with artificial mixtures of sugars and protein or amino acids gave no indication of interference by amino acids in the chromogen formation. The main difference between the artificial mixture and the native mucoprotein is the presence of free monosaccharides in the former and of glycosidically bound sugar residues in the latter. Of the mucoproteins investigated thyroglobulin gave an almost quantitative recovery of non-amino sugars, and BSM a substantially lower recovery. It would appear from Table 2 that a striking difference between the two mucoproteins is the amino sugar/non-amino sugar ratio. We have observed, as may be expected from the standard technique for liberating hexosamine (2 N-HCl, 6 hr., 100°), that under optimum hydrolysis conditions for non-amino sugars (0·25 N-$H_2SO_4$, 18 hr., 100°) only two-thirds of the hexosamine are released from the mucoprotein. With ovomucoid it was ascertained that at the end of the

mild hydrolysis the liberated amino sugar was completely deacetylated (see also Dixon, 1955), and it is probable that the same treatment would have also released all acetyl groups from the hexosamine still bound. Unless the hexosamine moiety left is a chitin-like structure, an appreciable proportion of the hexosamine residues will be linked glycosidically to non-amino sugars. The non-amino sugar trapped in this way will escape recovery, at step 5, both by the orcinol method and by chromatography. Such a situation would be analogous to the resistance towards acid hydrolysis of the glucosidic linkage in *N*-acetylmethylglucosaminide after deacetylation has taken place; the charged amino group, by repelling the approach of a proton to the glucosidic oxygen, confers an abnormal stability on methylglucosaminide towards acid (Moggridge & Neuberger, 1938). In mucoproteins the extent to which non-amino sugars will be trapped by this mechanism depends on the frequency of the structure hexosaminyl–non-amino sugar within the polysaccharide moiety. The trapping potentialities suggested by a high amino sugar/non-amino sugar ratio may be partly counterbalanced by a structure in which the hexosamine residues, linked mainly with each other, are engaged glycosidically in only small numbers with non-amino sugar. This may explain the similar percentage recovery by chromatography of the intrinsic non-amino sugars with BSM (68%) and ovomucoid (70%), although the ratios differ widely (4·41 and 1·71 respectively). It seems more than coincidence that, with thyroglobulin, with the previously studied urinary mucoprotein (Gottschalk, 1952*b*; Odin, 1952) and with a mucoprotein present in influenza virus (Ada & Gottschalk, 1956), where a recovery by chromatography of 90, 90 and 85% respectively of the non-amino sugars was achieved, the ratio was less than 1·0. It would thus appear that only where good agreement between the orcinol value of the original mucoprotein and the estimate of the component sugars, liberated by mild hydrolysis under optimum conditions, is obtained, will it be possible to establish the true proportion of the individual non-amino sugars.

With BSM there is a marked discrepancy between the values obtained by the orcinol and chromatographic methods when applied to the material carried through step 1 to step 4 (Table 1, columns 3 and 4). It was also observed that during mild hydrolysis of BSM considerable amounts of soluble and insoluble humin material were formed, only part of which was removed in the subsequent steps. The coloured material derives most probably from sialic acid, present to an extent of 17% in BSM (Werner & Odin, 1952) and known to decompose on mild acid treatment, with the production of humin matter (Blix, 1936). The soluble humin material and/or any other chromogen formed from sialic acid during hydrolysis (Gottschalk, 1955*b*) may react with orcinol, but being separated from the sugars on paper will not contribute to the chromatographic estimate.

Our results are not in agreement with the finding (Dixon, 1955) that after hydrolysis of ovomucoid with 0·5N-$H_2SO_4$ for 4 hr. at 100° only 30–40% of the material, estimated as sugar by application of the anthrone or orcinol method to the hydrolysate, can be recovered by chromatography. No difficulties were experienced in recovering by chromatography 70% of the intrinsic sugar, as determined by orcinol, when strict control of the pH throughout and maintenance of low temperatures during concentration were observed.

## SUMMARY

1. A method allowing the complete analysis of the sugar components of mucoproteins has been described. The main features of the method are the reduction to a minimum of the detrimental interaction between reducing sugars and amino acids before their separation and the chromatographic identification and determination of the individual sugars.

2. The accuracy of the method is ±10%. The recovery by the procedure of known amounts of sugar added to a protein is almost quantitative. The intrinsic sugars of a mucoprotein are recovered to an extent of 70–90%. One factor determining the degree of recovery appears to be the amino sugar/non-amino sugar ratio, a low value favouring a high recovery in the cases tested.

3. Within this limitation a complete analysis of the carbohydrate moiety of thyroglobulin, bovine-submaxillary-gland mucoprotein and ovomucoid is presented.

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# The Component Sugars of the Influenza-Virus Particle

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(*Received* 9 *September* 1955)

The great amount of information obtained over the last 15 years about the biological activities of the influenza virus (Burnet, 1951) stimulated at an early stage detailed biophysical and biochemical investigation of this organism. Thus the particle size and shape, electrochemical behaviour (Stanley, Knight & De Merre, 1945) and enzymic properties (Gottschalk, 1954) have been intensively studied. The virus particle is known to contain four main constituents: protein, lipid, carbohydrate and nucleic acid. Taylor (1944) found for the A strain (PR8) of influenza virus a total carbohydrate content of 12·5% (orcinol method with glucose as standard), made up, on the basis of the carbazole reaction, of mannose or glucose–galactose, or both. According to Knight (1947) the total carbohydrate of the PR8 strain, as determined by orcinol with glucose as standard, is about 7%. Of this, ribose was considered to constitute approximately one-tenth, the remainder being incorporated in a polysaccharide consisting of galactose, mannose and hexosamine, as indicated by the carbazole and Elson–Morgan reactions. The identification by Taylor and Knight of the constituent hexoses is based on a technique involving, for a mixed polysaccharide, the interpretation of superimposed absorption curves. The uncertainty of such an interpretation has been previously discussed (Gottschalk & Ada, 1956). For hexosamine no attempt at identification was made. The pentose nature of the nucleic acid sugar was ascertained by Bial's orcinol reaction, and its identity as ribose inferred from the observation that a sample of isolated nucleic acid was degraded by ribonuclease. Recently, the pentosenucleic acid content of the virus has been reinvestigated with improved techniques and a value of about 1% for highly purified and infective PR8 virus obtained; no significant amount of deoxyribosenucleic acid was found (Ada & Perry, 1954). In the preceding paper (Gottschalk & Ada, 1956), a method has been described allowing the identification and determination in mucoproteins and nucleic acids of the individual sugars after their separation from the non-carbohydrate moiety and after their chromatographic isolation. The desirability of having accurate information about the nature and amount of the sugar components of the influenza virus prompted a reinvestigation of the carbohydrate of the virus particle with the new technique.

## MATERIALS AND METHODS

*Influenza virus.* The strain A (PR8) was used throughout; it was purified by specific adsorption to and elution from human erythrocytes, followed by two cycles of differential centrifuging in an ultracentrifuge. The purity of the virus preparation was judged by examination in the electron microscope and assessed by determination of the ratio haemagglutinin units : mg. dry wt. Details have been described previously (Ada & Perry, 1954).

*Virus nucleic acid.* The defatted, dried virus was extracted with hot 10% (w/v) NaCl solution and the nucleic

acid precipitated by adding 2 vol. of ethanol to the extract. In view of the small amounts available the quantity of nucleic acid used in experiments was calculated from the molecular extinction coefficient (see below).

*Mucoprotein fraction from normal allantoic fluid.* Normal allantoic fluid was harvested from 12-day-old embryonated hen eggs. The liquid was clarified by centrifuging (2000 *g*, 15 min.), and the supernatant, after dialysis against distilled water for 48 hr. at 4°, dried from the frozen state *in vacuo*. The dried material was taken up in a minimum amount of water, redialysed and freeze-dried as above. When redissolved, this material, though free of reducing sugar, gave a positive Molisch test, and it was brought out of solution by protein precipitants (sulphosalicylic acid, trichloroacetic acid) and by heating.

*Ion-exchange resin.* Dowex 50 (200–400 mesh) was prepared as a hydrolysing agent as described by Dixon (1955).

*Determination of nucleic acid.* The nucleic acid was determined spectrophotometrically by applying the formula $\epsilon_P = 30{\cdot}98\ E/cl$, where $\epsilon_P$ represents the atomic extinction coefficient at 260 m$\mu$. with respect to phosphorus and found to be 9200 (Ada & Perry, in preparation), $E$ the optical density ($\log I_0/I$), $c$ the concentration of P in g./l. and $l$ the thickness of the absorbing layer (Chargaff & Zamenhof, 1948). The factor 9·7, calculated from the known proportion of bases (Ada & Perry, 1955*b*), was applied to convert the phosphorus value into nucleic acid concentration.

*Determination of total carbohydrate (non-amino sugar).* As shown earlier (Gottschalk & Ada, 1956), the orcinol method, when carried out with the correct standard, is satisfactory for the determination of total carbohydate in carbohydrate–protein complexes. In a few cases the total carbohydrate content was also estimated by the tryptophan method (Badin, Jackson & Schubert, 1953); values given by the two methods agreed within 5%. Each determination was carried out on 3–4 mg. of dried virus.

*Determination of component non-amino sugars.* The component sugars of 20–25 mg. of the dried virus were released by hydrolysis (0·25 N-$H_2SO_4$, 18 hr., 100°), unhydrolysed protein and liberated amino acids removed respectively by precipitation with ethanol and treatment with ion-exchange resin, and the purified sugars were isolated and quantitatively estimated by chromatography (Gottschalk & Ada, 1956). An average recovery of 80% (the orcinol value of the original viral material being taken as 100%) of the component sugars was obtained by this technique. This recovery must be regarded as very satisfactory, considering that about 5% of the orcinol-reacting material was left behind after hydrolysis in an insoluble fraction of the virus preparation.

The purine-bound sugar present in virus nucleic acid was liberated by hydrolysis with N-$H_2SO_4$ at 100° for 1 hr. and then processed as described previously (Gottschalk & Ada, 1956).

*Determination of amino sugars.* Hydrolysis of the virus preparation (3–4 mg.) and purification and estimation of the liberated amino sugars were carried out as reported elsewhere (Gottschalk & Ada, 1956).

*Identification of non-amino sugars.* The component sugars were identified by comparison with a set of standard sugars, run on paper in four suitable solvents (Gottschalk, 1955). Since the sugars found to be present in influenza virus were those most frequently occurring in carbohydrate–protein complexes, no sugar derivatives for identification by melting point were prepared.

*Identification of amino sugars.* The amino sugars were identified by their conversion into the corresponding pentoses, followed by chromatographic analysis (Gardell, Heljkenskjold & Rochnovlund, 1950). This identification procedure was checked by chromatographic separation of the purified hexosamines, a technique which also allowed an estimate of the approximate proportion of glucosamine and galactosamine (Gottschalk & Ada, 1956). About 5 mg. of dried virus was used for this estimation.

## RESULTS

*The sugar component of virus nucleic acid.* The purine-bound sugar present in virus nucleic acid, after liberation and purification, was chromatographed together with arabinose, xylose, lyxose and ribose in four different solvents (Gottschalk, 1955), all affording satisfactory separation, and a single spot obtained coinciding in each case with the ribose standard in both $R_F$ value and colour. Quantitative determination of the isolated ribose by the chromatographic method gave in a total of three experiments a mean value amounting to $92 \pm 9$% of the theoretical value calculated from the purine content of the virus nucleic acid. In a representative experiment on two portions (0·20 mg.) of nucleic acid the following values were obtained. In one portion 35 $\mu$g. of ribose was found; in the other, 20 $\mu$g. of adenine and 17 $\mu$g. of guanine, equivalent to 39 $\mu$g. of ribose (expressed as free pentose) were present. This result compares with that previously found for yeast nucleic acid (Gottschalk & Ada, 1956).

Since the quantity of virus nucleic acid available did not allow identification of the pyrimidine-bound pentose by its conversion into the corresponding acid (Levene & La Forge, 1912), an attempt was made to hydrolyse the *N*-glycosidic linkages with the hydrogen form of a sulphonated ion-exchange resin, conditions which would ensure the survival of the liberated sugar for an extended period (see Dixon, 1955). Virus nucleic acid (0·12 mg. in 0·20 ml. of water) was heated for 100 hr. at 100° in the presence of 25 mg. of resin. After separation of the resin the solution was concentrated to dryness at about pH 6 and the residue analysed by chromatography. The amount of sugar recovered and identified as ribose (as above) was 10 $\mu$g., accounting for no more than 25% of the theoretical calculated from the purine and pyrimidine base content (see above). This low figure would suggest that not only, as in mild acid hydrolysis, are the cytosine- and uracil-*N*-ribosides unaffected by the resin, but that even part of the purine-*N*-ribosides escapes hydrolysis with the latter as catalytic agent.

*Non-amino sugars (other than ribose) of the virus particle.* The total carbohydrate content of the virus particle, as estimated by the orcinol method, was found to vary between 3·0 and 5·6%, the mean value of the results from experiments with nine different virus batches being 4·6 (see Table 1). Of this amount, 6% is accounted for by ribose; the remainder is distributed between galactose, mannose and fucose in the proportion 10:6·4:5·3.

*Amino sugars of the virus particle.* The total amino sugar content of six different batches of influenza virus varied between 2·2 and 3·3%, with a mean value of 2·6%. Glucosamine was the only amino sugar (see Table 1).

In the six batches of virus in which both the non-amino sugar content and the amino sugar content were determined the value of the non-amino sugar: amino sugar ratio was calculated for each individual batch and found to vary between 2·0 and 1·1, with a mean value of 1·6.

*The carbohydrate moiety of the mucoprotein fraction of normal allantoic fluid.* The mucoprotein fraction prepared from normal allantoic fluid has been analysed, with the results tabulated.

## DISCUSSION

The evidence presented leaves little doubt that the pentose isolated from the purine nucleotides of the virus nucleic acid is ribose. The hope that the pentose linked to the pyrimidines might be detached by a cation-exchange resin was not substantiated. However, from the ribose nature of the purine-bound pentose of virus nucleic acid, from the ribose identity of the purine- and pyrimidine-bound pentose in yeast nucleic acid (Levene & La Forge, 1912) and from the identity of the bases in yeast ribose and virus pentose nucleic acid (Ada & Perry, 1955*a*), it seems most likely that the pyrimidine bases of virus nucleic acid are also linked to ribose.

The spectrum of the remainder of the virus carbohydrate, consisting of galactose, mannose, fucose and glucosamine, closely resembles that of neutral mucoproteins (Gottschalk & Ada, 1956). Indeed, no heteropolysaccharide of such a composition is known to exist in nature unless bound to protein. Though the proportion of the individual non-amino sugars is remarkably constant, the total amount of non-amino sugar and of amino sugar varies appreciably and independently from batch to batch of virus. The variation in the ratio non-amino sugar:amino sugar may reflect the variation from virus to virus in the carbohydrate composition of a single mucoprotein or the presence in the virus particle of two or more different mucoproteins.

Table 1. *Analysis of the carbohydrate of the influenza-virus particle and of the mucoprotein fraction of the normal allantoic fluid*

The percentage values given are expressed as anhydro sugars. The virus figure in the last column is not computed from the means as given in columns 2 and 7, but is calculated from the figures of each individual batch of virus.

| Material | Total carbohydrate content* (non-amino sugar) (%) | Ribose content† (%) | Proportion of non-amino sugars | | | Total hexosamine content (%) | Proportion of amino sugars | | Non-amino sugar* / Amino sugar |
|---|---|---|---|---|---|---|---|---|---|
| | | | Galactose | Mannose‡ | Fucose‡ | | Glucosamine | Galactosamine | |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) |
| Influenza-virus particle | 4·6±1·0§ (9)‖ | 0·28±0·04 (3) | 10·0 (6) | 6·4±0·6 (6) | 5·3±0·5 (6) | 2·6±0·4 (6) | 10 (3) | 0 (3) | 1·6±0·3 (6) |
| Mucoprotein fraction of allantoic fluid | 3·8 | 0 | 10 | 15 | 8 | 2·8 | 10 | 1 | 1·4 |

* Orcinol value.
† Evaluated from the ribose recovered from the purine nucleotides and assuming the ribose nature of the pyrimidine-bound pentose.
‡ Referred to galactose.
§ Standard deviation $= \sqrt{\frac{\Sigma(\bar{x}-x)^2}{n-1}}$.
‖ Figures in brackets refer to the number of experiments.

As may be seen from Table 1, the mucoprotein fraction of the allantoic fluid, which derives from potential host cells of the virus, contains the same sugars as are present in the virus particle. This observation, taken together with the variation of the carbohydrate content of the virus, raises the question whether or not the mucoprotein present in the virus preparation is a contaminant or an integral part of the particle. The nature of the virus purification process, including specific adsorption to and elution from red cells, eliminates the possibility of a contaminant unassociated with the virus particle. A contaminant associated with the virus by adsorption to its surface seems to be excluded on quantitative grounds; even with a low protein content (e.g. 50%) such a contaminant would entirely cover the particle surface, a situation incompatible with the known enzymic and antigenic properties of the virus. The mucoprotein is therefore considered to be an integral part of the virus particle. It remains to be determined, however, whether the virus exerts a genetic control over the pattern and amount of the mucoprotein or whether its presence in the virus is merely a necessary concomitant of the process by which new virus particles are produced. Whether gene-controlled or not, the general resemblance of the virus mucoprotein to that produced by the non-infected cell would suggest that the same basic mechanism operates in both cases.

## SUMMARY

1. The purine-bound pentose of influenza virus nucleic acid has been identified chromatographically as ribose.

2. The remainder of the virus carbohydrate, consisting of galactose, mannose, fucose and glucosamine, is built into a polysaccharide considered to be the carbohydrate moiety of a mucoprotein.

3. Though the proportion of the component non-amino sugars is fairly constant, the total amounts of non-amino sugar and of amino sugar vary appreciably and independently.

4. The potential host cells of the virus were found to produce mucoprotein with a carbohydrate moiety of similar composition to that of the virus.

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# Liver Glycerylphosphorylcholine Diesterase

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(*Received* 26 *September* 1955)

Recent isotopic experiments have led to the conclusion that free glycerylphosphorylcholine (GPC) and glycerylphosphorylethanolamine (GPE) occur in liver tissue as intermediates in the catabolism of phosphatidylcholine and phosphatidylethanolamine respectively (Dawson, 1955). This being so, it appeared likely that an enzyme would be present in the tissue which was capable of further hydrolysing these phosphodiesters. The present paper describes some of the properties and distribution of such an enzyme, which catalyses the hydrolysis of the diesters into glycerophosphoric acid and the free base. Some of the preliminary observations on the enzyme were made in collaboration with Mr A. Bowyer in 1954.

Contardi & Ercoli (1933) reported that the product obtained by the action of lecithinase B on lecithin was acted on by a further enzyme present in kidney, intestine and pancreas, which liberated choline. The enzyme concerned, however, appears to differ from the present enzyme in having a pH optimum of 4 (cf. 7·5) and in being inhibited by

eserine. Shapiro (1952, 1953) found that extracts from acetone-dried pancreas were able to convert lecithin into GPC but that no further hydrolysis occurred. Recently, Hayaishi & Kornberg (1954) have described the presence of a GPC diesterase in a strain of *Serratia plymuthicum* grown on a lecithin-enriched medium; the enzyme appears to differ from that in liver only by its sensitivity to magnesium ions and by its pH optimum.

The investigation of GPC diesterase in liver necessitated the preparation of the substrate GPC in quantity. As stated by Aloisi & Buffa (1948), earlier published methods for doing this succeeded only in preparing the choline salt of glycerophosphoric acid. In 1948, Baer & Kates published an elegant chemical method for preparing the optically active ester, and recently Uziel & Hanahan (1955) have reported a preparation in which lecithin is treated with a lecithinase present in extracts from *Penicillium notatum*.

Baer & Kates (1950) found that during the alkaline hydrolysis of lecithin the fatty acids were liberated at a faster rate than choline, and deduced from the hydrolysis rates that GPC was an intermediate. This was confirmed chromographically by Dawson (1954), who found that, after a brief alkaline hydrolysis of lecithin in methanolic NaOH, GPC was formed (yield, 84%). In the present investigation these observations have been made the basis of a method for preparing GPC from ovolecithin.

A preliminary report of this work was given to the Second International Congress on Biochemical Problems of the Lipids, Ghent, 1955.

## EXPERIMENTAL

*Preparation of* L-α-*glycerylphosphorylcholine.* Ovolecithin used in the preparation of GPC was prepared by the method of Hanahan (1954) with an activated alumina column to fractionate the crude egg-yolk phospholipids. The ovolecithin (1·4 g.), which consisted of an off-white waxy solid, was dissolved in 8 ml. of carbon tetrachloride, and to the solution was added 63 ml. of methanol and 9 ml. of water. The solution was treated with 20 ml. of M methanolic NaOH and incubated at 37° for 10 min. The hydrolysate was cooled and diluted with 150 ml. of water and the milky liquid rapidly passed (10–15 min.) through a column of Amberlite IRC 50 (H) resin (20 cm. × 3 cm.²; analytical grade; large beads). The Na-free effluent, plus washings, was shaken in succession with equal volumes of carbon tetrachloride, ether, light petroleum, and finally *iso*butanol, which cleared any residual turbidity from the solution. The solution was then filtered and adjusted to pH 7·5 with $NH_3$ and reduced in volume *in vacuo* to approximately 4 ml. After filtering, the clear solution was treated with 100 ml. of acetone. A milky turbidity immediately formed, and on standing overnight or longer at −10° the acetone could be easily decanted away from an opalescent oil clinging to the walls of the flask. The oil was dissolved in 40 ml. of cold absolute ethanol; the solution was filtered clear of insoluble matter and the GPC recovered by removing the ethanol by distillation under reduced pressure and finally drying the residue in a vacuum desiccator over $CaCl_2$. This gave a colourless viscous oil (yield, 74% based on the lecithin used). {Found: $[\alpha]_D^{22}$, −2·5°; P, 11·1; hydrolysable choline, 42·0 (Entenman, Taurog & Chaikoff, 1944); α-glycerol ester, 100·3% (Voris, Ellis & Maynard, 1940). L-α-GPC requires $[\alpha]_D$, −2·85°; P, 11·27; hydrolysable choline, 44·0; α-glycerol ester, 100%.} It is clear from these results that little migration of the phosphate group could have occurred during the brief incubation with alkali.

Some preparations of the diester showed on paper chromatography in phenol–$NH_3$ traces of GPE, undoubtedly arising from phosphatidylethanolamine impurity in the lecithin. It was found that this could readily be removed by preparing the $CdCl_2$ complex of GPC (Baer & Kates, 1948) and recrystallizing this from ethanol–water.

The GPC was stored in ethanolic solution and kept at −20°.

*Enzyme preparation.* GPC diesterase was studied in extracts prepared from acetone-dried rat liver. The preparation of these extracts needed to be carefully done, otherwise low or negligible activities were obtained. The liver from one rat was frozen in a mortar cooled to −20°, and then broken down into small pieces. Ice-cold water (2 vol.) was then slowly added, the mixture being vigorously rubbed with the pestle in order to keep the suspension as homogeneous as possible. The mixture was kept at 0° for 15 min. and then refrozen. It was allowed to thaw while being vigorously rubbed with the pestle. Acetone at −20° (5 vol.) was then added and the suspension immediately filtered under reduced pressure. The filter cake was immediately washed with a further 10 vol. of acetone (−20°) and finally with cold ether. It was dried free from solvent in a vacuum desiccator over liquid paraffin and solid NaOH. The slightly off-white powder was rubbed up in a mortar with 4 vol. of ice-cold water, and dialysed overnight against 100 vol. of distilled water at 0°. The suspension was spun at 8000 *g* for 12 min. and the clear supernatant collected. It could be stored frozen for several days with only a slight loss of activity.

*Enzyme assay.* GPC-diesterase activity was estimated by measuring the choline liberated in an incubation medium which contained 0·25 ml. of enzyme preparation: 0·5 ml. of Na-glycylglycine buffer (0·1 M, pH 7·8 at 20°); 0·1 ml. of $MgCl_2$ (0·01 M); 0·1 ml. of GPC (0·02 M) and 0·05 ml. of water. Incubation of the enzyme with and without substrate was carried out for 30 min. at 37°. At the end of this period the reaction mixture was cooled in ice and then treated with 0·5 ml. of ice-cold 9% (w/v) perchloric acid. The precipitated proteins were removed by centrifuging and 1 ml. samples of the supernatant assayed for free choline.

*Estimation of choline.* Choline was estimated in the supernatant by using the periodide method of Appleton, La Du, Levy, Steele & Brodie (1953). The preliminary purification of the sample was omitted and 0·2 ml. of the cold potassium triiodide reagent was added directly to the supernatant. Throughout the entire estimation, including the colorimetry, it was found necessary to keep all solutions at 0°. With dialysed enzyme preparations the blanks were very low, and the recovery of added choline was practically linear up to 32 μg., the maximum tested. Precipitation of the proteins with trichloroacetic acid or

tungstic acid was not found to be as satisfactory as with perchloric acid. With homogenates the recovery of choline added in small concentrations tended to be low.

*Miscellaneous.* Chromatographic methods used for the examination of phosphate esters have been described in previous papers (Dawson, 1954, 1955). Phenol was estimated by the method formulated by King (1946). Ovolecithin was prepared by the method of Hanahan (1954). A sample of L-α-GPE was kindly provided by Professor E. Baer (Toronto). Lysolecithin was a gift from Drs C. H. Lea and D. N. Rhodes (Cambridge). Sodium diphenylphosphate was a gift from Albright and Wilson Ltd.

## RESULTS

In initial experiments it was found that when homogenates of rat liver were incubated with L-α-GPC at 37° there was a rapid liberation of free choline. The activity was practically all located in the supernatant fraction after spinning for 10 min. at 8000 *g*. However, owing to the difficulty of estimating choline in such homogenates the investigation was continued with extracts from acetone-dried powders. When such extracts were freshly prepared, and incubated with GPC according to the procedure described in the Experimental section, approximately 60–80 μg. of choline was liberated per ml. of incubation medium in 30 min. The activity of the enzyme slowly declined on incubation, and it was found that on preincubating the extract for 1 hr. at 37° only about 50% of its former activity was retained.

*Reaction products.* The accumulation of choline in the incubation medium was confirmed by its precipitation as the reineckate. No liberation of inorganic phosphate occurred when this ion was estimated by the method of Fiske & Subbarow (1925). Chromatography of the phosphate esters in phenol–$NH_3$ and ethanol–$NH_3$ showed that the disappearance of α-GPC was associated with an accumulation of α-glycerophosphoric acid in the incubation medium.

*Effect of metals.* Dialysis of the extract from acetone-dried rat liver overnight caused a small decrease in activity (16–24%). The addition of $Mg^{2+}$ at $10^{-3}$M slightly stimulated (16–26%) activity, both in the undialysed and dialysed extracts, but this stimulation became an inhibition with higher concentrations of $Mg^{2+}$ (Fig. 1). $Mn^{2+}$ added at the same concentration ($10^{-3}$M) slightly reduced the activity, and $Ca^{2+}$ (54%) and $Zn^{2+}$ (90%) were even more inhibitory. Ethylenediaminetetraacetic acid (EDTA) at a concentration of $10^{-3}$M virtually abolished activity.

pH *optimum.* Fig. 2 shows that the pH optimum of GPC diesterase, measured in a series of buffers (phosphate, glycylglycine and glycine) was approximately 7·5. Below pH 6·5 or above 9·0 little activity was observed. These results were also confirmed in a series of glycylglycine buffers. It should be noted that the pH of a sodium glycylglycine buffer falls fairly rapidly with temperature, and consequently a buffer showing a pH of 7·8 at 20° was used in the enzyme-assay system.

*Effect of inhibitors.* Fluoride ($10^{-2}$M) and iodoacetate ($2{\cdot}5 \times 10^{-3}$M) caused no appreciable diminution of enzyme activity. The specific inhibitors of true and pseudocholinesterase, di*iso*propylphosphorofluoridate (DFP, $10^{-5}$M) and eserine ($2 \times 10^{-5}$M) did not inhibit GPC-diesterase activity.

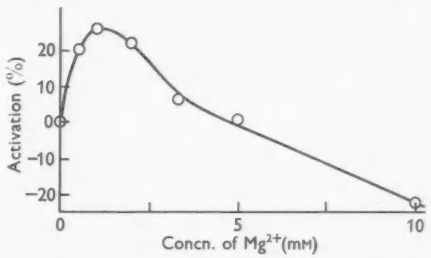


Fig. 1. Effect of $Mg^{2+}$ on the GPC-diesterase activity of an acetone-dried liver extract (dialysed). Activity measured at 37° in 0·045M sodium glycylglycine buffer (pH 7·8 at 20°).

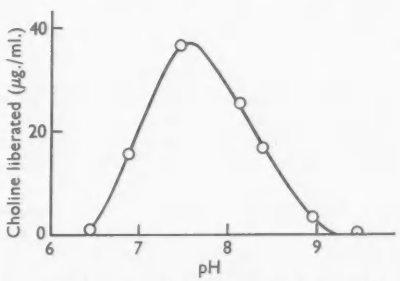


Fig. 2. Variation of GPC-diesterase activity with the pH of the incubation medium. Activity measured at 37° with the following buffers: pH 6·45, sodium phosphate (0·11M); pH 6·88, 7·45 sodium glycylglycine (0·275M); pH 8·14, 8·38, 8·96, 9·47 sodium glycine (0·055M).

The strong inhibitory action of EDTA and $Zn^{2+}$ has been mentioned in the section on the effect of metals.

*Substrate specificity.* Free choline was not liberated from phosphorylcholine, lysolecithin or lecithin under the conditions used for enzyme assay. Lecithin was not attacked in the presence of ether, which activates the lecithinase that hydrolyses choline from lecithin (Kates, 1953). When L-α-GPE was incubated with the enzyme preparation

hydrolysis of the diester occurred. L-α-GPE was also an inhibitor of GPC hydrolysis and Lineweaver & Burk (1934) plots showed that the inhibition was competitive (Fig. 3). Phenol was liberated from diphenylphosphate at a slow rate (10% of GPC hydrolysis), but the hydrolysis was slightly inhibited by fluoride. The enzyme preparation showed a very small phosphomonoesterase activity measured at pH 7·5 with β-glycerophosphate as the substrate, but this was completely abolished by the addition of fluoride (0·02 M).

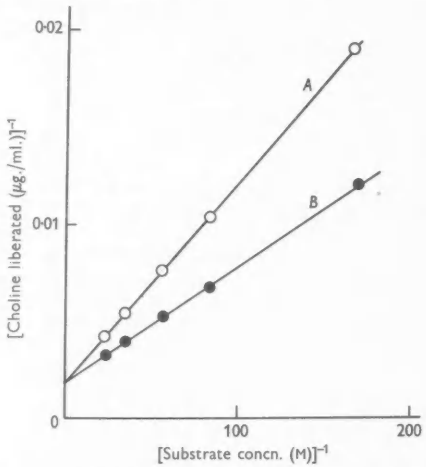


Fig. 3. Influence of substrate concentration on liver-GPC-diesterase activity in the presence of two concentrations of L-α-GPE: (*A*) 0·007 M; (*B*) 0·0035 M. Activity measured at 37° in 0·045 M sodium glycylglycine buffer (pH 7·8 at 20°).

*Other tissues.* Three samples of liver tissue which were collected from sheep soon after slaughter were found to have very low or negligible GPC-diesterase activities when assayed in a similar manner to that used for rat liver. All other rat tissues which were examined as homogenates showed appreciable ability to hydrolyse GPC (brain, heart, kidney, spleen, intestine, skeletal muscle and whole blood). Four samples of rat plasma and one of ox plasma showed no activity. On the other hand, the corpuscular fraction of rat blood showed appreciable activity.

## DISCUSSION

Experiments reported in this paper show that an enzyme is present in rat-liver extracts which hydrolyses L-α-glycerylphosphorylcholine into α-glycerophosphoric acid and free choline. The same liver extract also breaks down L-α-glycerylphosphorylethanolamine, and, as this diester was found to be a competitive inhibitor of GPC hydrolysis, it seems reasonable to assume that a single enzyme is responsible.

It is known that GPC and GPE are not attacked by phosphatases (Schmidt, Greenbaum, Fallot, Walker & Thannhauser, 1955), and in fact the present enzyme preparation showed very little phosphomonoesterase activity, especially in the presence of fluoride, an inhibitor which does not affect GPC diesterase. The ability of the preparation to hydrolyse GPC was not reduced in the presence of eserine or DFP, and therefore the enzyme does not fit the currently accepted definition of a true or pseudocholinesterase. Moreover, highly active preparations of these enzymes isolated from ox plasma by Dr D. C. Hardwick showed no ability to hydrolyse GPC.

A comparison of the present enzyme with the GPC-splitting enzyme recently found to occur in *Serratia plymuthicum* grown on a lecithin-enriched medium (Hayaishi & Kornberg, 1954) indicates two differences: the liver enzyme is stimulated by the addition of $Mg^{2+}$ ($10^{-3}$ M), whereas a similar concentration of this ion almost abolishes the activity of the bacterial enzyme. The bacterial enzyme also has a higher pH optimum (9); at this pH the liver enzyme shows very little activity.

It seems very likely that the physiological importance of GPC diesterase in liver and other tissues of the rat is connected with phosphoglyceride metabolism. Isotopic experiments have shown the liver to be one of the most active phospholipid-synthesizing tissues, and this would suggest that appreciable phosphoglyceride catabolism must also occur in the tissue. One of the pathways of phosphatidylcholine and phosphatidylethanolamine catabolism *in vivo* appears to be their hydrolysis to form fatty acids and GPC or GPE (Dawson, 1955). The present results indicate that these diesters are subsequently hydrolysed in the tissue into glycerophosphoric acid and the free base. Calculation from isotopic results has shown that approximately 23 mg. of phosphatidylcholine and 26 mg. of phosphatidylethanolamine are broken down via this route per hour in 100 g. of normal rat liver (Dawson, 1956).

The apparent lack of GPC-diesterase activity in sheep liver may help to explain the high concentrations of GPC and GPE which are found in the liver of this species (Schmidt, Hecht, Fallot, Greenbaum & Thannhauser, 1952; Schmidt *et al.* 1955).

## SUMMARY

1. A method has been developed for the rapid chemical preparation of L-α-glycerylphosphorylcholine (GPC) from ovolecithin.

2. An enzyme has been found in extracts prepared from acetone-dried rat liver which catalyses

the hydrolysis: L-α-GPC + $H_2O$ → α-glycerophosphoric acid + choline.

3. Similar extracts also attacked L-α-glycerylphosphorylethanolamine, and this diester was a competitive inhibitor of GPC hydrolysis.

4. The GPC-splitting activity of such extracts was maximal at pH 7·5 and was stimulated by the addition of $Mg^{2+}$ ($10^{-3}$ M). Similar concentrations of $Mn^{2+}$ and $Ca^{2+}$ were inhibitory and this also applied to higher concentrations of $Mg^{2+}$.

5. The enzyme was not sensitive to fluoride, iodoacetate, eserine or di*iso*propylphosphorofluoridate. However, ethylenediaminetetraacetic acid and $Zn^{2+}$ were strong inhibitors.

6. Evidence has been obtained for the presence of the enzyme in other tissues of the rat, but it is virtually absent from the liver of the sheep. Activity was not observed in rat plasma, but it was found in the blood corpuscles.

The author is indebted to Sir Rudolph Peters, F.R.S., for his interest and encouragement during this investigation. He thanks Miss Norma Hemington for her very valuable assistance.

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# Studies on the Phosphorylcholine of Rat Liver

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(*Received* 26 *September* 1955)

The recent isolation of phosphorylcholine in considerable quantities from aqueous extracts of rat liver (Dawson, 1955*a*) has raised the question of its origin in the tissue *in vivo* and its possible role as an intermediary in lecithin synthesis. Two enzymes are known which can produce phosphorylcholine in tissues: a lecithinase which splits lecithin into phosphorylcholine and a diglyceride, and choline phosphokinase, which catalyses the direct phosphorylation of choline by adenosine triphosphate (Wittenberg & Kornberg, 1953). With regard to its possible role as a lecithin precursor, recent experiments of Rodbell & Hanahan (1955), following the work of Kornberg & Pricer (1952), have convincingly demonstrated that phosphorylcholine can be incorporated as a unit into the lecithin of guinea-pig liver mitochondrial preparations.

Experiments have therefore been performed whereby radioactive phosphate was administered to rats, and a study made of the rate of labelling of both liver phosphorylcholine and lecithin. The results indicate that phosphorylcholine is not formed in the tissue through the action of a lecithinase, and the rapid rate with which it becomes labelled with $^{32}P$ suggests some process such as the direct phosphorylation of choline. At the same time examination of the specific radioactivity time curves using the Zilversmit, Entenman & Fishler (1943) predictions does not indicate an immediate precursor–product relationship between liver phosphorylcholine and lecithin.

In further experiments, designed to obtain information about the metabolic relationships of phosphorylcholine with other choline-containing compounds, the level of the substance in rat liver has been compared with that of liver glyceryl-

phosphorylcholine (GPC) during various conditions such as starvation, insulin hypoglycaemia, and after choline administration.

## EXPERIMENTAL

Experimental methods used in the present report have been fully described in a previous paper (Dawson, 1955*b*). Two small improvements, however, were introduced into the procedure for obtaining two-dimensional chromatograms of the water-soluble P-containing compounds of the liver. The solution was freed from cations with an Amberlite IRC 50 column, and ethanol–$NH_3$ was used as the second solvent in the paper chromatography. The location and identification of the phosphorylcholine spot on such chromatograms has been described previously (Dawson, 1955*a*); the ethanol–$NH_3$ solvent used instead of propanol–$NH_3$ did not alter the relative position of the spots but resulted in a greater separation of phosphorylcholine and glycerylphosphorylcholine.

Animals used for the estimation of phosphorylcholine and glycerylphosphorylcholine levels in the liver were killed by a blow on the neck, and the liver was rapidly dissected and plunged into liquid $O_2$ (<1 min.). The frozen tissue was then crushed and a two-dimensional chromatogram prepared of the water-soluble phosphorus-containing compounds in the tissue. The phosphorus contained in the spots was estimated in the usual way after complete oxidation of the paper with perchloric acid (Dawson, 1955*b*). Constant recoveries of approximately 75% were obtained when synthetic glycerylphosphorylcholine and phosphorylcholine were added to tissue extracts, and carried through the entire experimental procedure. Although therefore the chromatographic recoveries are not quantitative, in the absence of any other suitable methods of estimation, they do give a good indication of the relative levels of these phosphoesters in the tissue.

## RESULTS

Fig. 1 compares the specific radioactivities of rat-liver phosphorylcholine and lecithin in a series of animals killed at various times after the intraperitoneal injection of a constant amount of $^{32}P$ as phosphate. Immediately after the injection the phosphorylcholine becomes rapidly labelled with $^{32}P$, and its specific radioactivity rises well above that of the liver lecithin. For example, 100 min. after injection the phosphorylcholine has a specific radioactivity which is at least 20 times greater than that of liver lecithin. This precludes the possibility that the phosphorylcholine occurs in the tissue as an intermediary in the catabolism of lecithin. For comparison, included on Fig. 1 are data on the specific radioactivity of liver glycerylphosphorylcholine, a compound which is now known to arise in the tissue by the breakdown of lecithin (Dawson, 1955*b*). Although the specific radioactivity of liver phosphorylcholine is initially higher and subsequently lower than that of the lecithin, it does not appear to obey the Zilversmit *et al.* (1943) criteria for being the immediate phosphorus precursor of the phosphoglyceride; its specific radioactivity does not fall to equal that of the lecithin until some hours after the latter has reached its maximum specific radioactivity (Fig. 1).

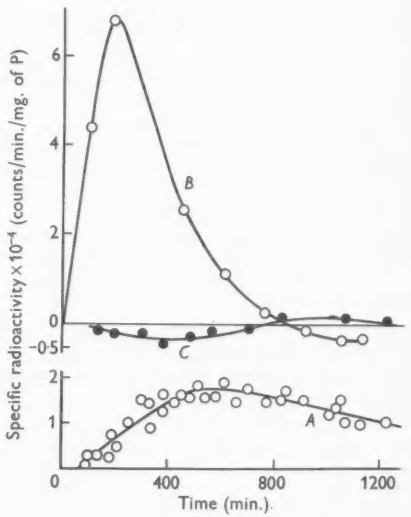


Fig. 1. Curves showing (*A*) the specific radioactivities of liver lecithin; (*B*) the specific radioactivities of liver phosphorylcholine minus that of lecithin; (*C*) the specific radioactivities of liver glycerylphosphorylcholine minus that of lecithin; at various times after the injection of labelled phosphate ($2 \times 10^6$ counts/min./100 g. of body wt.).

In Table 1 are recorded the recoveries of phosphorylcholine and glycerylphosphorylcholine obtained on paper chromatography of extracts from the livers of rats killed during various physiological states. As is recorded in the experimental section, these recoveries are only approximately 75% quantitative, and they represent minimal concentration values only. Experiments were performed with series of four animals, one of which was always a control allowed free access to food and water. In these control rats the molar concentration of phosphorylcholine was usually 3–4 times greater than that of glycerylphosphorylcholine and in one animal was 8 times greater. In animals killed 45–90 min. after the intraperitoneal injection of a single dose of choline chloride (200 mg./kg.) the recovery of phosphorylcholine was always greater than that from the control animal (26–74% increase), and that of the glycerylphosphorylcholine was unaltered. On the other hand, in four rats killed after 24 hr. starvation the mean recovery of glycerylphosphorylcholine from the liver was over twice that found in the livers of control

animals, and at the same time the phosphorylcholine content of the tissue did not increase.

A further series of rats were made hypoglycaemic by the intraperitoneal injection of insulin. It was found that appreciable doses of the hormone were needed before the animals showed visible signs of the hypoglycaemia, presumably because they had not been starved before the injection.* In all these animals, the amounts of glycerylphosphorylcholine and phosphorylcholine recovered from the liver increased markedly (Table 1). Even in rats where the hypoglycaemia was not sufficient to cause the animals to have convulsions or to become comatosed, the recoveries were still well above those of the control values.

Table 1. *Chromatographic recoveries of phosphorylcholine and glycerylphosphorylcholine (GPC) from rat liver during starvation and insulin hypoglycaemia and after the injection of choline*

The concentrations expressed represent minimal values only.

| | Phosphorylcholine (μmoles/g.) | GPC (μmoles/g.) |
|---|---|---|
| Controls | | |
| (6 rats) Means ± S.E. | 1·52 ± 0·14 | 0·35 ± 0·04 |
| Range | (1·15–1·91) | (0·22–0·53) |
| Choline chloride injection (200 mg./kg. body wt.) | | |
| (5 rats) Means ± S.E. | 2·14 ± 0·15 | 0·39 ± 0·04 |
| Range | (1·90–2·70) | (0·29–0·55) |
| | $P < 0·02$ | — |
| Starvation (24 hr.) | | |
| (4 rats) Means ± S.E. | 1·34 ± 0·14 | 0·82 ± 0·14 |
| Range | (1·02–1·76) | (0·48–1·12) |
| | — | $P < 0·01$ |
| Insulin hypoglycaemia | | |
| (5 rats) Means ± S.E. | 2·81 ± 0·14 | 1·46 ± 0·17 |
| | $P < 0·01$ | $P < 0·01$ |
| Rat injected with 40 units of insulin at 150, 60 and 30 min. before killing. Activity slightly depressed | 2·46 | 1·17 |
| Rat injected with 26 units of insulin at 145 and 30 min. before killing. Activity depressed | 2·85 | 1·64 |
| Rat injected with 80 units of insulin at 150 and 30 min. before killing. Semi-comatose | 2·60 | 1·66 |
| Rat injected with 90 units of insulin at 270, 180 and 150 min. before killing. In convulsions | 3·24 | 1·04 |
| Rat injected with 80 units of insulin at 145 and 20 min. before killing. In convulsions | 2·96 | 1·76 |

## DISCUSSION

It must be emphasized that the present isotopic results merely suggest that phosphorylcholine is not the sole immediate phosphorus precursor of liver lecithin, and not that the ester plays no part in lecithin synthesis *in vivo*. Indeed such participation seems highly likely in view of the recent demonstration of its incorporation *in vitro* as a unit into the liver lecithin of mitochondrial preparations (Rodbell & Hanahan, 1955). Kennedy & Weiss (1955) have recently published preliminary evidence which suggests that phosphorylcholine is converted by liver mitochondria into 'cytidine diphosphate choline' ($P^1$-cytidine-5′-$P^2$-choline-pyrophosphate) before it participates in lecithin synthesis. However, the departure of the phosphorylcholine and lecithin radioactivity–time curves from the expected precursor–product relationship predicted by Zilversmit *et al.* (1943) cannot be explained by the assumption that phosphorylcholine is first converted into another intermediate like 'cytidine diphosphate choline', as such a compound would obey the criteria even less readily than phosphorylcholine. Thus the specific radioactivity of the choline P in such an intermediary would fall to equal that of the liver lecithin at a time later than for phosphorylcholine. The failure to obey the Zilversmit *et al.* criteria may be connected with the recent demonstration that another pathway of lecithin synthesis, independent of phosphorylcholine as an intermediary, exists in liver mitochondria (Kennedy, 1954; Kennedy & Weiss, 1955). The breakdown of the Zilversmit *et al.* (1943) criteria in a metabolic system in which a compound is synthesized from more than one precursor has recently been emphasized in a theoretical paper by Reiner (1953).

* Subsequent work has shown that rats allowed food *ad lib.* can tolerate 1000 units of insulin/kg. body wt. injected intraperitoneally.

As the present results support the view that liver choline can be rapidly phosphorylated *in vivo*, it seems likely that the level of phosphorylcholine will to some extent be a reflexion of the relative rates of formation and further metabolism of free choline. On the other hand, the GPC content can be expected to depend on the rate of lecithin breakdown occurring in the tissue, as this compound has now been identified as an intermediate in the catabolism of liver lecithin (Dawson, 1955*b*). Any GPC liberated from liver lecithin would be subsequently hydrolysed into free choline and glycerophosphoric acid under the action of the enzyme GPC diesterase present in liver tissue (Dawson, 1956).

Thus in the present investigation the injection of choline into the rat was associated with an expected increase in the phosphorylcholine level without alteration of the GPC content. Starvation, which in the mammal produces a rapid loss of phosphoglycerides from the liver (Kosterlitz, 1947; Hodge *et al.* 1948), causes a marked rise in the GPC content, presumably because of the extra lecithin catabolism, and it might therefore be expected that the phosphorylcholine level would also rise. That such an increase does not occur during starvation may be related to the absence of dietary choline and methionine intake, and the requirements of the animal for these substances for transmethylation reactions. In insulin hypoglycaemia the level of GPC in the tissue is markedly increased, and this, as is to be expected, is associated with an elevation of the phosphorylcholine content.

## SUMMARY

1. Specific radioactivities of liver phosphorylcholine and lecithin have been measured at various times after the injection of radioactive phosphate into the rat.

2. The results indicate that phosphorylcholine is not formed in the liver by the breakdown of lecithin, nor does it appear to obey the Zilversmit *et al.* (1943) radioactive criteria for the sole immediate phosphorus precursor of liver lecithin.

3. Chromatographic recovery of phosphorylcholine in the liver is increased after the injection of choline but not after starvation.

4. Conversely, the recovery of liver glycerylphosphorylcholine is increased after starvation but is unaltered by choline administration.

5. Chromatographic recoveries of both liver phosphorylcholine and glycerylphosphorylcholine are markedly increased during insulin hypoglycaemia.

The author is indebted to Sir Rudolph Peters, F.R.S., for his interest and encouragement during this investigation. He thanks Miss Norma Hemington for her very valuable assistance.

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# Enzymic Properties of Microsomes and Mitochondria from Silver Beet

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(*Received* 14 *October* 1955)

Extensive studies on cells of animal origin from a wide variety of tissues have already established the localization of enzymic activity in cytoplasmic particles. The enzymic activities of mitochondria from animal tissues indicate that these structural units are sites of respiratory activity (see Schneider, 1953). A more limited series of studies have shown that the mitochondria from plant cells have a similar function (Millerd & Bonner, 1953; Goddard & Stafford, 1954). However, in neither plant nor animal cells is there as yet any clear indication of the metabolic function of the submicroscopic cytoplasmic particles (so-called 'microsomes'), although a number of enzymic activities appear to

be associated with them (see Hogeboom, Schneider & Striebich, 1953; Morton, 1954*a*, *b*; Bailie & Morton, 1955; Martin & Morton, 1955).

Stafford (1951) studied the intracellular localization of a selected number of enzymes in pea seedlings, but did not establish the occurrence of any of these enzymes in the small particles. However, the chemical analyses showed certain differences between the large- and small-granule fractions. Brown, Jackson & Chayen (1953) and Robinson & Brown (1953) established that small particles (20–37 m$\mu$. diameter) could be isolated from disrupted bean-root cells. They also showed an increase in ribonucleic acid (RNA) content with decrease of particle size. Other workers (Newcomb & Stumpf, 1952; Bokman, Newcomb, Castelfranco & Stumpf, 1953) have described a fatty acid oxidase system which they believe to be localized in the small 'yellow particles' insolated from peanut. Webster (1953) noted that the enzymic breakdown of glutathione by hypocotyls from bean seedlings was partly associated with 'microsomes'.

This and subsequent papers are concerned with the characterization of plant microsomes by investigation of their enzymic activities, chemical composition, size and structure. It was hoped that such studies would provide a basis for determining whether or not microsomes have any metabolic function in the plant cell. It should be stressed that the term 'microsome' is used to designate a particle as isolated from disrupted tissues. This does not necessarily imply that a particle as such exists in the intact cell. Studies relating to the origin of microsomal particles will be reported in a later paper.

The isolation and enzymic characterization of microsomes from the white petiole of silver beet (*Beta vulgaris*) is described in this paper, together with observations on the comparative enzymic properties of beet mitochondria.

This work was described at the 31st meeting of the Australian and New Zealand Association for the Advancement of Science, Melbourne, in August 1955.

## MATERIALS AND METHODS

*Diphosphopyridine nucleotide* (*DPN*). This was a commercial preparation (cozymase 90, Sigma Chemical Co.). It was reduced enzymically, with crystalline ethanol dehydrogenase prepared from yeast according to Racker (1950). Reduced DPN (DPNH) as supplied by C. F. Boehringer and Soehne, Mannheim, Germany, was also used.

*Triphosphopyridine nucleotide* (*TPN*). This product was 80% pure and it was reduced enzymically with glucose 6-phosphate dehydrogenase. (Both were obtained from Sigma Chemical Co.)

*Cytochrome* c. This was prepared from ox heart by the procedure of Keilin & Hartree (1945). Reduced cytochrome *c* was prepared as described by Margoliash (1952).

*Other substrates.* Commercial A.R. grade chemicals were used.

*Antimycin A.* This was a gift from Dr F. M. Strong and Dr G. M. Tener, University of Wisconsin. The antimycin A was dissolved in 95% ethanol, to give a stock solution (500 $\mu$g./ml.), from which suitable dilutions for the inhibition studies were prepared in distilled water. Similar dilutions of 95% (v/v) ethanol were used in the controls.

*Phosphate buffer.* A stock solution containing 42·4 g. of $NaH_2PO_4,2H_2O$ and 238·4 g. of $K_2HPO_4$/l. (0·8 M, pH 7·4) was prepared and suitably diluted for use. Unless otherwise specified, the term 'phosphate buffer' refers to this preparation.

### *Determination of enzymic activities*

*Dehydrogenases.* Activities were determined by measuring the rate of reduction of 2:6-dichlorophenolindophenol at 600 m$\mu$., using a Beckman spectrophotometer (Model DU). Measurements were made in 1 cm. cuvettes at room temp. (approx. 18–22°) in phosphate buffer (pH 7·4; final concn. 0·2 M for succinic dehydrogenase and 0·066 M for all other dehydrogenases). The final volume in each cuvette was 3·0 ml. and the final concn. of dye was $7 \times 10^{-5}$ M. The concns. of substrates for the various activities were: for succinic dehydrogenase, 0·06 M sodium succinate; for DPNH- or TPNH-diaphorases, $1 \times 10^{-4}$ M DPNH or reduced TPN (TPNH); for choline dehydrogenase, 0·02 M choline hydrochloride; for lactic dehydrogenase, 0·05 M sodium DL-lactate; for hypoxanthine dehydrogenase, approx. 0·01 M hypoxanthine.

*Cytochrome* c *reductases.* The rate of reduction of cytochrome *c* at 550 m$\mu$. was measured essentially as described by Slater (1950). The final concns. of reactants were: 0·066 M phosphate buffer (pH 7·4); 0·001 M potassium cyanide; $1 \times 10^{-4}$ M oxidized cytochrome *c*; for $DPNH_2$ and TPNH-cytochrome *c* reductases, $1 \times 10^{-4}$ M DPNH or TPNH; for succinate–cytochrome *c* reductase, 0·06 M sodium succinate.

*Cytochrome* c *oxidase.* The rate of oxidation of reduced cytochrome *c* ($1 \times 10^{-4}$ M) in phosphate buffer (0·066 M, pH 7·4) was measured spectrophotometrically at 550 m$\mu$. The solutions used were equilibrated with air.

*Fumaric hydrogenase.* The method used was a modification of that of Fischer, Roedig & Rauch (1942). The assay system comprised: phosphate buffer (pH 7·4, 0·1 M), Janus Green B ($5 \times 10^{-4}$ M), sodium fumarate (0·066 M), and sodium dithionite ($Na_2S_2O_4$, 1·0 to $1·2 \times 10^{-3}$ M). The substrate, buffer, dye and enzyme were mixed in the main compartment of a Thunberg tube. After evacuation and refilling with oxygen-free nitrogen, the sodium dithionite (in phosphate buffer) was tipped from the side arm and the time for reoxidation of the reduced dye to safranin was measured. The amount of enzyme and of dithionite was adjusted so that the time for reoxidation was between 2 and 3 min. A control tube (without fumarate) was handled simultaneously, a T-joint being used to ensure the same gas phase. The time for reoxidation in the control tube was always greater than 20 min. The results were expressed as $\mu$moles of sodium dithionite oxidized/hr./mg. of N. All measurements were made at 38°. In order to use a slight excess of dithionite in the reaction, it was anaerobically titrated against the amount of Janus Green B added. The amount of dithionite/tube was determined by standardization against potassium ferricyanide.

*Chemical method*

Nitrogen was determined colorimetrically with Nessler's reagent, after digestion with sulphuric acid as previously described (Morton, 1955).

## EXPERIMENTAL

*Choice of tissue.* In preliminary studies, succinic-dehydrogenase activity was used as an index of the suitability of any tissue. It was assumed that metabolically active plant material (from which microsomes might be obtained) should show appreciable succinic-dehydrogenase activity. Moreover, since this enzyme is particularly labile in plants (Price & Thimann, 1951), those procedures which give high succinic-dehydrogenase activity in the tissue dispersion should also be suitable for preservation of enzymic activity in microsomes. With this in mind, and because of limited facilities for growing plants, a survey of a number of readily available plant tissues was undertaken.

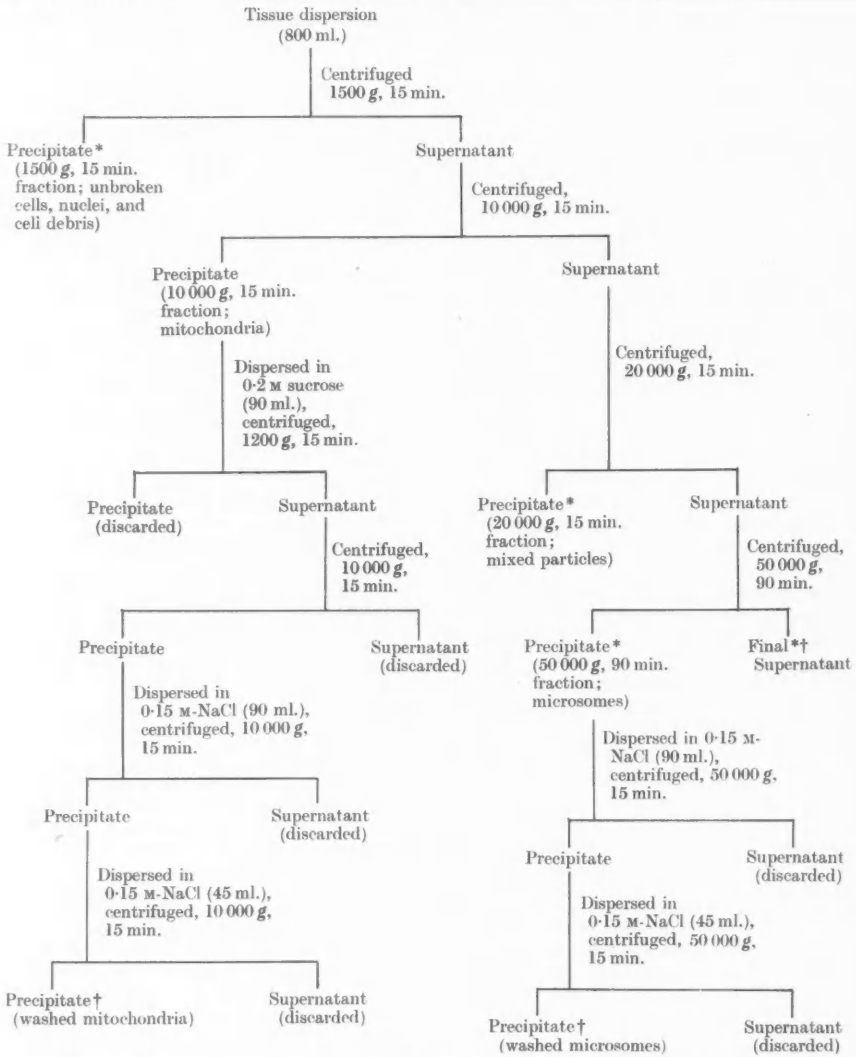
Tissue dispersion (800 ml.)
Centrifuged 1500 g, 15 min.
Precipitate* (1500 g, 15 min. fraction; unbroken cells, nuclei, and cell debris)
Supernatant
Centrifuged, 10 000 g, 15 min.
Precipitate (10 000 g, 15 min. fraction; mitochondria)
Supernatant
Dispersed in 0·2 M sucrose (90 ml.), centrifuged, 1200 g, 15 min.
Centrifuged, 20 000 g, 15 min.
Precipitate (discarded)
Supernatant
Precipitate* (20 000 g, 15 min. fraction; mixed particles)
Supernatant
Centrifuged, 10 000 g, 15 min.
Centrifuged, 50 000 g, 90 min.
Precipitate
Supernatant (discarded)
Precipitate* (50 000 g, 90 min. fraction; microsomes)
Final*† Supernatant
Dispersed in 0·15 M-NaCl (90 ml.), centrifuged, 10 000 g, 15 min.
Dispersed in 0·15 M-NaCl (90 ml.), centrifuged, 50 000 g, 15 min.
Precipitate
Supernatant (discarded)
Precipitate
Supernatant (discarded)
Dispersed in 0·15 M-NaCl (45 ml.), centrifuged, 10 000 g, 15 min.
Dispersed in 0·15 M-NaCl (45 ml.), centrifuged, 50 000 g, 15 min.
Precipitate† (washed mitochondria)
Supernatant (discarded)
Precipitate† (washed microsomes)
Supernatant (discarded)

Fig. 1. Fractionation of silver beet petiole dispersion. *, Fractions used for enzymic analyses; †, fractions used for chemical analyses.

Preliminary experience with different tissues (sugar-beet root, soya-bean seedlings, carrot root, cauliflower inflorescence, radish root, celery stem and white petiole of silver beet) showed that a satisfactory level of succinic-dehydrogenase activity was to be found in the tissue dispersions of celery, beet petiole and soya-bean seedlings. Initially, celery was chosen for these studies since it had the highest succinic-dehydrogenase activity of the more readily available tissues. Unfortunately, this high activity was found to vary considerably during the short growing season, and celery stem was abandoned in favour of silver-beet petiole, which showed less seasonal variation.

*Disruptive procedure.* The fragility of plant chloroplasts is well established. Damage results in the appearance of chlorophyll in the supernatant and concomitant reduction in the size of the sedimented-chloroplast layer. Using this criterion, supported by microscopic examination of the isolated fractions, a number of different disruptive techniques were studied. With the green leaf and green petiole of silver beet, it was found that hand grinding of the tissue in a mortar, without the addition of an abrasive, gave most satisfactory cell breakage without appearance of chlorophyll in the supernatant. That disruption of the cell occurred without accompanying damage to the cytoplasmic particle was also confirmed by finding that the succinic-dehydrogenase activity sedimented almost entirely in the fraction obtained at 10 000 *g* for 15 min. It should be stressed, however, that cell breakage cannot be considered as quantitative.

*Dispersion medium.* The hypertonic (0·88 M) sucrose solution of Hogeboom, Schneider & Pallade (1947) was initially chosen for isolation of enzymically active particulate components. However, this was abandoned in favour of 0·2 M sucrose, since the latter gave higher succinic-dehydrogenase activities in isolated mitochondria. The effect of various protective agents (egg albumin, bovine serum albumin, lactoglobulin, starch and peptone) on the succinic-dehydrogenase activity of the final particulate preparation was investigated. Slight enhancement of activity was obtained with media containing both egg albumin and peptone. Since the tissue dispersion in sucrose solution alone was distinctly acid, potassium phosphate buffer (0·2 M) at pH 7·4 was included in the medium. For simplicity and lack of interference with later studies, nitrogenous protective agents were omitted and a medium containing 0·2 M sucrose and 0·2 M potassium phosphate, adjusted to pH 7·4, was used.

*Isolation of particles.* Leaves and stem from young silver-beet plants were harvested on the morning of the preparation. The white petiole was dissected free of all leaf tissue and washed thoroughly with cold water. All ensuing operations were carried out in a cold room at about 2°. The petioles were chopped by hand into pieces approx. 0·5 cm. × 0·5 cm. × 0·1 cm. About 100 g. of chopped pieces was ground for approx. 2 min. with 150 ml. of the above 0·2 M sucrose and phosphate medium, in a large earthenware mortar and pestle previously cooled to about 0°. The dispersion was strained through four thicknesses of muslin cheese cloth. Usually about 400 g. of tissue was used for each preparation, the final volume of the dispersion being about 750–800 ml.

It was observed in preliminary studies that the succinic-dehydrogenase activity, used as a guide to the localization of mitochondria, accompanied the particles sedimented at 10 000 *g* for 15 min. Fractionation of the tissue dispersion was therefore carried out as shown in Fig. 1. In order to reduce contamination of the microsomal fraction with mitochondria, an intermediate fraction (mixed particles) was isolated by centrifuging at 20 000 *g* for 15 min.

The first fraction (1500 *g*, 15 min.) was obtained at 0° with an International refrigerated centrifuge fitted with an angle head. All other fractions were obtained with a Servall vacuum-type centrifuge (Model SS 2), the angle head being cooled to about 0° before use. The temp. rose to about 5° during isolation of the 10 000 *g* fraction, and to about 10° during isolation of the other fractions.

The time elapsing from the dispersion of the tissue to the sedimentation of the microsomal fraction (50 000 *g*, 90 min.) was normally about 6–7 hr. Washing and refractionating required an additional 5 hr. In order to minimize loss of enzymic activity, samples were normally removed as soon as sedimented and used for enzymic determinations (see Fig. 1). The remainder was then washed as shown in Fig. 1 (to remove contaminating soluble constituents and dispersion medium) and used for chemical analyses, which will be described in a later paper.

## RESULTS

### *Enzymic activities of fractions isolated from beet petiole*

The succinic-dehydrogenase, DPNH-cytochrome *c* reductase, and DPNH-diaphorase activities were estimated for each of the isolated fractions. These

Table 1. *Enzymic activities of fractions from beet-petiole dispersion*

The fractions were isolated by differential centrifuging of tissue dispersions from beet petiole and activities were determined as described in the text. Mean activities and ranges are given for four different preparations. Activities are expressed as μmoles of acceptor reduced/hr./mg. of N.

| | Enzymic activities | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Succinic dehydrogenase | | DPNH-cytochrome *c* reductase | | DPNH-diaphorase | | Ratio of activities | | |
| Fraction | Mean | Range | Mean | Range | Mean | Range | *a** | *b*† | *c*‡ |
| 1500 *g*, 15 min. (unbroken cells, etc.) | 1·1 | 0·0–2·9 | 8·0 | 3·4–14·0 | 40·0 | 33·0–47·0 | 7 | 37 | 5 |
| 10000 *g*, 15 min. (mitochondria) | 1·2 | 0·9–1·6 | 16·2 | 12·0–21·0 | 55·0 | 51·0–59·0 | 14 | 47 | 3 |
| 20000 *g*, 15 min. (mixed particles) | 0·4 | 0·0–0·6 | 23·3 | 12·0–33·6 | 22·6 | 22·0–24·0 | 58 | 57 | 1 |
| 50000 *g*, 90 min. (microsomes) | 0·1 | 0·0–0·2 | 39 | 27·0–55·0 | 49·0 | 43·0–55·0 | 390 | 490 | 1 |

* $\frac{\text{DPNH-cytochrome } c \text{ reductase}}{\text{Succinic dehydrogenase}}$. † $\frac{\text{DPNH-diaphorase}}{\text{Succinic dehydrogenase}}$. ‡ $\frac{\text{DPNH-diaphorase}}{\text{DPNH-cytochrome } c \text{ reductase}}$.

activities have been determined on a large number of different preparations over a period of about 18 months, during which time a considerable variation in total activity of the petiole dispersions has been observed. However, the relative activities of the different fractions have always been essentially the same. Table 1 summarizes the results of four representative experiments which were carried out over a period of 10 days. It can be seen that the ratios of the various enzymic activities of the isolated fractions show a distinct change in enzymic pattern with decrease of particle size.

*Distribution of enzymes in dispersions of beet petiole*

Table 2 shows the distribution of the total nitrogen and of the succinic-dehydrogenase, DPNH-cytochrome *c* reductase and DPNH-diaphorase activities in the various fractions isolated from beet petiole. Results are expressed as a percentage of the total activity of the original dispersion. The results shown in Table 2 are representative of results obtained in two different experiments. Good recoveries of activities were obtained except in the case of succinic dehydrogenase, for which several repetitions gave no greater recovery than 20% of the activity of the dispersion.

*Effect of antimycin A on enzymic activities of beet petiole*

Ahmad, Schneider & Strong (1950) and Potter & Reif (1952) showed that the antibiotic antimycin A is a powerful inhibitor of succinic dehydrogenase and of DPNH-cytochrome *c* reductase of rat liver. However, as yet there have been no comparable studies with plant tissues.

Experiments were carried out with mitochondria and microsomes isolated from beet petiole by the following modification of the standard procedure:

Small quantities (50–100 g.) of petiole were dispersed in the medium as previously described. The dispersion was centrifuged at 1500 *g* for 15 min. at 0°. The supernatant was recentrifuged at approx. 10000 *g* for 20 min. at 0° with the high-speed attachment of the International refrigerated centrifuge. The sediment (mitochondria) was resuspended in 0·15 M-NaCl, pH 7·4, at 0°. The supernatant was recentrifuged at 60000 *g* for 1 hr. in a Spinco centrifuge, Model L, at 0°. The precipitate (microsomes) was suspended in 0·15 M-NaCl, pH 7·4, at 0°.

In this way, cytoplasmic particles were prepared within 3 hr. of disruption of the tissue, as compared with 8 hr. by the standard procedure.

Table 3 shows that mitochondrial succinate- and DPNH-cytochrome *c* reductase activities are inhibited by antimycin A, whereas the microsomal DPNH-cytochrome *c* reductase activity is unaffected. The higher activities of both the mitochondria and microsomes as shown in Table 3, as

Table 2. *Distribution of nitrogen and enzymic activities among fractions from beet-petiole dispersion*

The fractions were isolated by differential centrifuging of tissue dispersions from beet petiole. Each fraction was suspended in a known volume of 0·2 M sucrose solution for chemical and enzymic estimations, carried out as described under Methods. Activities are expressed as for Table 1, except for fumaric hydrogenase. This activity is expressed as $\mu$moles of $Na_2S_2O_4$ oxidized/hr./mg. of N.

| Fraction | Nitrogen | | Succinic dehydrogenase | | Fumaric hydrogenase | | DPNH-cytochrome *c* reductase | | DPNH-diaphorase | |
|---|---|---|---|---|---|---|---|---|---|---|
| | mg./100 g. fresh weight | % whole dispersion | Activity | % whole dispersion | Activity | % whole dispersion | Activity | % whole dispersion | Activity | % whole dispersion |
| Whole dispersion | 43·8 | 100 | 0·22 | 100 | 0·17 | 100 | 0·76 | 100 | 9·3 | 100 |
| 1500 *g*, 15 min. (unbroken cells, etc.) | 3·55 | 8·1 | 0·19 | 7·0 | 0·17 | 8·1 | 1·63 | 17·4 | 20·2 | 17·6 |
| 10000 *g*, 15 min. (mitochondria) | 3·00 | 6·8 | 0·34 | 10·6 | 0·26 | 10·5 | 3·55 | 32·3 | 18·8 | 13·6 |
| 20000 *g*, 15 min. (mixed particles) | 1·04 | 2·4 | 0 | 0 | 0 | 0 | 6·8 | 21·1 | 15·5 | 3·9 |
| 50000 *g*, 90 min. (microsomes) | 1·57 | 3·6 | 0 | 0 | 0 | 0 | 7·1 | 33·7 | 22·9 | 8·8 |
| Supernatant | 31·6 | 72 | 0 | 0 | 0 | 0 | 0 | 0 | 8·1 | 62·0 |
| Total | — | 93 | — | 18 | — | 19 | — | 105 | — | 106 |

Table 3. *Effect of antimycin A on succinate- and DPNH-cytochrome c reductases of beet-petiole mitochondria and microsomes*

Fractions were isolated from beet-petiole dispersion by a modification of the standard procedure, designed to preserve enzymic activities by reducing the preparative time from about 8 to 3 hr. (see text, p. 700). The reactions were started by the addition of substrate. Activities were measured as described under Methods and are expressed as $\mu$moles of cytochrome *c* reduced/hr./mg. of N.

| Fraction | Substrate | Antimycin A* concn. $\mu$g./mg. of nitrogen | Enzymic activities: Control | Enzymic activities: With antimycin A | % inhibition |
|---|---|---|---|---|---|
| 10 000 *g*, 20 min. (mitochondria) | Succinate | 5 | 11·2 | 0·86 | 93 |
| | | 250 | 12·9 | 0·13 | 99 |
| | Reduced DPN | 0·25 | 13·8 | 13·8 | 0 |
| | | 0·5 | 13·3 | 11·6 | 13 |
| | | 1·0 | 16·3 | 12·1 | 26 |
| | | 2·5 | 13·3 | 9·0 | 32 |
| | | 5·0 | 15·0 | 9·9 | 34 |
| | | 25 | 14·2 | 9·2 | 35 |
| | | 250 | 16·0 | 10·2 | 36 |
| 60000 *g*, 60 min. (microsomes) | Reduced DPN | 60 | 70·0 | 70·0 | 0 |
| | | 300 | 68·0 | 68·0 | 0 |

* The highest concn. of antimycin A used in each case (mitochondria and microsomes) corresponded to 3 $\mu$M in the cuvette.

Table 4. *Other enzymic activities of beet mitochondria and microsomes*

Mitochondria (10 000 *g*, 15 min. fraction) and microsomes (50 000 *g*, 90 min. fraction) were prepared as described in the text. Enzymic activities were determined as described under Methods, and are expressed as $\mu$moles of acceptor reduced/hr./mg. of N. The results are representative activities from different preparations.

| Enzymic activity | Mitochondria | Microsomes |
|---|---|---|
| TPNH-cytochrome *c* reductase | 5·4 | 25 |
| TPNH-diaphorase | 21·7 | 12·4 |
| Hypoxanthine dehydrogenase | 0·6 | 2·0 |
| Choline dehydrogenase | 0·2 | 0·7 |
| Cytochrome *c* oxidase | 2·6 | 0·2 |
| Lactic dehydrogenase | 0·7 | Nil |

compared with the activities shown in Tables 1 and 2, are undoubtedly due to the more rapid preparative procedures used in this experiment.

*Other enzymic activities*

In order to investigate whether particulate cytoplasmic components of beet petiole may have associated enzymic activities other than the three enzymes mentioned in Table 1, a survey was carried out with substrates other than succinate or reduced DPN in the dehydrogenase-assay system. Table 4 lists enzymes found in the mitochondrial or microsomal fractions with definite detectable activities. In addition, the following enzymes were shown to be absent or of an activity too low to be estimated accurately: adenosine triphosphatase, inorganic pyrophosphatase, glycylglycine dipeptidase, glycylleucine dipeptidase and methyl butyrate esterase.

## DISCUSSION

Claude (1938, 1944) described 'microsomes' as submicroscopic particles isolated from liver by differential centrifuging of the dispersed tissue. Subsequently, Kabat (1941) demonstrated that alkaline phosphatase was associated with submicroscopic particles from kidney which sedimented only at high centrifugal force. A considerable amount of evidence has since been obtained by various authors which permits characterization of microsomes from animal tissues on the basis of their size, characteristic enzymic activities and unique chemical composition (see Hogeboom *et al.* 1953; Morton, 1954*a*, *b*).

Isolation of submicroscopic particulate material alone in no way suffices to characterize microsomes. It has been repeatedly demonstrated that submicroscopic lipoprotein particles, comparable to microsomes in size and sedimentation characteristics, may be isolated by disruption of mitochondria from animal tissues (Green, 1952; Cleland & Slater, 1953). While the observations of a number of authors, as noted in the introduction, suggest that microsomes may occur in plant tissues, in no case hitherto has the characterization been sufficient to justify the use of this term.

It seems likely that most of the cytoplasmic material obtained in this work originated from the cortical cells of the beet petiole, with some contribution from the phloem and other cells.

*Occurrence of microsomes in plant cells*

The evidence presented establishes that the particles sedimented between 20 000 and 50 000 *g* (see Fig. 1) correspond essentially to the 'micro-

some' fraction already well-characterized for animal cells.

The sedimentation characteristics, supported by electron-microscope observations (Hodge, Martin & Morton, unpublished work) show that the particles fall in the size range 30–300 mμ. Moreover, they are quite distinct from plant mitochondria both in size and structure. As indicated above, such submicroscopic particles quite possibly could be obtained as artifacts produced by disruption of the plant cell. However, the preparative procedure used caused no substantial damage to fragile cytoplasmic components such as chloroplasts or mitochondria. Accordingly, these may be disregarded as sources of the submicroscopic particles in this investigation. The evidence (Hodge *et al.* unpublished work) for the origin of the plant microsomes from a primitive type of endoplasmic reticulum will be presented in a subsequent paper.

Particularly striking is the unique enzymic constitution of the submicroscopic particles, as compared with that of mitochondria. This is very clearly indicated by consideration of the ratios of enzymic activities shown in Table 1. Whereas the ratio of DPNH-cytochrome *c* reductase to succinic dehydrogenase is about 14 for mitochondria, it is about 390 for the microsomes. Corresponding differences are seen in the ratios of the other enzymic activities. Although the recovery of succinic-dehydrogenase activity is low (Table 2) there is no reason to believe that the loss of activity preferentially occurred from any one isolated fraction.

The results shown in Table 2 suggest that no enzymic activity is exclusively localized in the microsome fraction. This is misleading, however, since the DPNH-cytochrome *c* reductase activities of mitochondria and of microsomes clearly belong to different enzymic systems. They are differentiated on the basis of sensitivity to antimycin A (Table 3). Whereas the microsomal activity is completely insensitive to antimycin A, even at very high concentrations (3 μM, 300 μg. of antimycin A/mg. of nitrogen), the mitochondrial DPNH-cytochrome *c* reductase activity is inhibited by very low levels (0·006 μM, 0·5 μg. of antimycin A/mg. of nitrogen) of antimycin A.

The contention that the DPNH-cytochrome *c* reductase activity of the microsomal fraction is distinct from that of mitochondria is supported by the finding that the reductase activity of the microsome fraction is intimately associated with cytochrome $b_3$ (Martin & Morton, 1955). Studies of the distribution of haem proteins in the isolated fractions have shown that cytochrome $b_3$ is exclusively localized in the microsomes (Martin & Morton, unpublished). TPNH-cytochrome *c* reductase activity is greater in beet microsomes than in mitochondria (Table 4). Like DPNH-cytochrome *c* reductase activity, it is also associated with cytochrome $b_3$ in the microsomes and is not inhibited by antimycin A (Martin & Morton, 1955). It should be noted that cytochrome *c* is used merely as a convenient acceptor for hydrogen atoms from reduced DPN (and reduced TPN), in order to differentiate the activity from that of DPNH-(and TPNH-)diaphorase. While cytochrome *c* is probably the natural acceptor for the mitochondrial DPNH- (and TPNH-)cytochrome *c* reductases, it may be excluded as a component of the microsomal system (Martin & Morton, 1955).

The enzymic evidence thus leaves no doubt that the isolated submicroscopic particles have associated metabolic activities distinct from those of mitochondria or other cytoplasmic fractions. Chemical analyses, which will be reported in a subsequent paper, also reveal differences between the mitochondrial and microsomal fractions.

### *Enzymic activities of mitochondria from beet petiole*

The mitochondria obtained from the beet petiole closely resemble those isolated from other plant tissues, such as mung bean, avocado pear and cauliflower inflorescence (see Bonner & Millerd, 1953; Goddard & Stafford, 1954). As found by other workers with plant tissues (Price & Thimann, 1951; Laties, 1953, 1954) the succinic dehydrogenase of beet mitochondria is particularly labile. The low recovery of activity (Table 2) appears to be largely attributable to a continual decline of activity during the prolonged isolation procedure. This procedure was necessary in order to isolate sufficient material to carry out both enzymic and chemical studies. In other experiments, such as that reported in Table 3, in which the preparative period was greatly shortened, the succinic-dehydrogenase activity of the isolated mitochondria was considerably higher.

The components of the succinic oxidase system in plants have not, as yet, been isolated or identified. Like mitochondria from animal tissues, those from silver beet petiole contain considerable amounts of cytochrome *b*, which has been implicated in the succinate-cytochrome *c* reductase system (Keilin & Hartree, 1949; Slater, 1949). However, the succinic dehydrogenase of heart muscle is not identical with cytochrome *b* (Morton, 1950, and unpublished work; see Chance & Smith, 1952). Slater (1949) has suggested that a labile factor mediates between the cytochrome *b* and cytochrome *c* components of the succinate-cytochrome *c* reductase system of animal tissues. This component appears to be similar to a factor specifically inhibited by antimycin A (Ahmad *et al.* 1950; Potter & Reif, 1952; Chance, 1952). The very

strong inhibition of the succinate-cytochrome *c* reductase activity by low concentrations of antimycin A (Table 3) suggests that a component similar to the Slater factor is involved in the reduction of cytochrome *c* by succinic dehydrogenase of plant tissues.

It was shown by Fischer and co-workers (Fischer & Eysenbach, 1937; Fischer *et al.* 1942) that yeast contains an enzyme which catalyses reduction of sodium fumarate in the presence of sodium dithionite and suitable dyes. The enzyme, called 'fumaric hydrogenase', was partially purified and shown to be a flavoprotein. The relationship of fumaric hydrogenase to succinic dehydrogenase of heart muscle was investigated by Harrison & Morton (unpublished work), who showed that the soluble, partially purified succinic dehydrogenase (Morton, 1950) also had associated hydrogenase activity. Subsequent studies (Morton, unpublished work) have confirmed that fumaric-hydrogenase activity is manifested by a component of the heart-muscle succinic-dehydrogenase system. It is therefore of considerable interest that the distribution of fumaric-hydrogenase activity of beet petiole closely followed the distribution of succinic dehydrogenase (Table 2). As in the case of succinic dehydrogenase, the total recovery of activity was low (about 20% of that of the original dispersion). These findings confirm the intimate relationship of fumaric-hydrogenase and succinic-dehydrogenase activities.

Slater (1950) proposed that the DPNH-cytochrome *c* reductase system of heart muscle comprised several components and could be represented by the following scheme:

Reduced DPN → diaphorase → factor → cytochrome *c*,

where the arrows represent the direction of hydrogen transfer. This scheme involves the same factor (sensitive to antimycin A) as that implicated in the succinate-cytochrome *c* reductase system (see p. 702). Whereas the mitochondrial succinate-cytochrome *c* reductase is almost completely inhibited by antimycin A (Table 3), the mitochondrial DPNH-cytochrome *c* reductase, while sensitive to low levels of antimycin A, is only partially inhibited (Table 3). It seems very unlikely that the finding of an inhibition of only 36% at high concentrations of antimycin A is due to the occurrence in mitochondria of the antimycin A-resistant DPNH-cytochrome *c* reductase of microsomes. Rather, the results suggest that the coupling of reduced DPN to cytochrome *c* via the Slater factor is not as complete in plant mitochondria as it is in animal mitochondria, in which the system is more strongly inhibited by antimycin A (Chance, 1952; Pappenheimer & Williams, 1954).

*DPNH-diaphorase activity of plant tissues*

Considerable interest still attaches to the relationship between DPNH-diaphorase and DPNH-cytochrome *c* reductase. As indicated above, Slater (1950) proposed that diaphorase and an additional factor were components of the DPNH-cytochrome *c* reductase system in heart muscle. Loss of the factor linking the flavoprotein to cytochrome *c* would account for diaphorase activity without associated DPNH-cytochrome *c* reductase activity.

The results in Table 2 show that in plants the DPNH-diaphorase and DPNH-cytochrome *c* reductase activities are not necessarily related. While all the DPNH-cytochrome *c* reductase activity of the beet petiole is associated with particulate material, most of the DPNH-diaphorase activity (62%) remains in the final supernatant fraction. In view of the good recovery of activity, it seems unlikely that the DPNH-diaphorase activity of the supernatant is due to damage to the particulate components with consequent release of enzyme to the supernatant. The evidence suggests that the total DPNH-diaphorase activity of the petiole dispersion is the resultant of at least three different systems. Mitochondrial DPNH-cytochrome *c* reductase and microsomal DPNH-cytochrome *c* reductase may both exhibit some dye reductase (diaphorase) activity, while a third and distinct DPNH-diaphorase, not associated with cytochrome *c* reductase activity, occurs in the supernatant fraction. The significance of this latter enzyme in plant metabolism is quite obscure at the present time.

*Function of plant microsomes*

Recent evidence indicates that the microsome fraction, derived from the endoplasmic reticulum of liver, is directly concerned in the uptake of amino acids and probably in protein synthesis in liver (Keller, Zamecnik & Loftfield, 1954). The findings presented here and elsewhere (Martin & Morton, 1955; Bailie & Morton, 1955) leave little doubt that microsomes from plants contain enzymic systems analogous to those of microsomes from animal tissues. It therefore seems probable that the microsome fraction of plants, like that of animal tissues, is also concerned in protein synthesis.

## SUMMARY

1. White petiole of silver beet (*Beta vulgaris*) was used to obtain cytoplasmic components with minimal disruption of mitochondria or plastids. The particulate components were isolated by differential centrifuging of the tissue dispersion.

2. Succinic-dehydrogenase, DPNH-cytochrome *c* reductase and DPNH-diaphorase activities were

determined on all fractions. The relative enzymic activities in the microsomal fraction were different from those in the mitochondrial fraction.

3. The DPNH-cytochrome *c* reductase activity of beet microsomes is not inhibited by antimycin A, while the similar activity of the mitochondria is sensitive to very low concentrations of this antibiotic.

4. The succinate-cytochrome *c* reductase activity of beet mitochondria is almost completely inhibited by low concentrations of antimycin A. This suggests that a factor, similar to the Slater factor, is concerned in the succinate-cytochrome *c* reductase system of plants.

5. Fumaric-hydrogenase activity closely parallels that of succinic dehydrogenase, both in cytoplasmic localization and in lability.

6. At least three different systems appear to contribute to the total DPNH-diaphorase activity of beet-petiole dispersions. Over 60% of the diaphorase activity occurs in the 'soluble' supernatant fraction, and all the DPNH-cytochrome *c* reductase is associated with particulate material.

The financial assistance of the Commonwealth Scientific and Industrial Research Organization, in the form of a Studentship for one of us (E.M.M.), is gratefully acknowledged.

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